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Investigations into the Pathogenesis of Essential Thrombocythaemia

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A thesis submitted to the University of London for the degree Doctor of Philosophy

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ABSTRACT

Essential thrombocythaemia (ET) is a chronic myeloproliferative disorder of unknown aetiology, characterized by sustained thrombocytosis and megakaryocyte hyperplasia. Three strategies were used to investigate disease pathology. Firstly, PCR-based assays using fluorescently labelled primers to measure X-chromosome inactivation patterns were evaluated and clonality status shown to be stable in 14 patients with polyclonal and 8 patients with clonal myelopoiesis studied over a median of 54 months (range 10-102) and 52 months (range 5-97) respectively. Secondly, mutations and polymorphisms in cytokines implicated in thrombopoiesis were investigated in ET patients. No mutations in the 5' untranslated region of the thrombopoietin gene, previously described in hereditary thrombocythaemia, were found. Genotypes and gene frequencies for polymorphisms previously shown to affect circulating cytokine levels in the transforming growth factor β1 and interleukin-6 genes did not differ between patients and controls. Thirdly, representational difference analysis was used to compare global gene expression in platelet mRNA from an ET patient with monoclonal myelopoiesis with that from a normal control. Three genes were identified as differentially expressed: RANTES, CD32 (FcγIIRA), and FLP. CD32 expression was further investigated using a multiplex semi-quantitative RT-PCR assay. Platelet CD32 expression was increased in 52 ET patients compared to normal controls (median CD32:GAPDH ratio 21, range 2-73 and 1, range <1-3 respectively, $p=0.005$). However, expression levels were not significantly different between 15 polyclonal and 18 clonal patients (median ratios 23, range 2-73, and 25, range 8-60, respectively, $p=0.281$), nor between 11 polycythaemia vera patients and eight patients with reactive thrombocytosis (median ratios 30, range 7-63, and 10, range 2-25, respectively). Therefore, whilst this cohort of ET patients has raised levels of CD32 mRNA, this cannot currently be used as a diagnostic marker, or to identify patients at higher risk of thrombotic complications.

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ABBREVIATIONS

(a+l) TGFβ	active and acid inducible latent transforming growth factor β
AChE	acetylcholinesterase
AML	acute myeloid leukaemia
AMM	agnogenic myeloid metaplasia
APA	antiphospholipid antibodies
AUG	transcription initiation site
BTK	bruton tyrosine kinase
blastn	nucleotide-nucleotide BLAST search
BM	bone marrow
BMT	bone marrow transplant
bFGF	basic fibroblast growth factor
BFU-MK	burst forming unit megakaryocyte
CAMT	congenital amegakaryocytic thrombocytopenia
CFU	colony forming unit
CFU-L	colony forming unit lymphoid
CFU-MK	colony forming unit megakaryocyte
cIMF	chronic idiopathic myelofibrosis
CML	chronic myeloid leukaemia
cMPD	chronic myeloproliferative disorder
CRH	cytokine receptor homology domain
DMS	demarcation membrane system
DPM	disintegrations per minute
DP (1)(2)	difference product (1)(2)
EEC	endogenous erythroid colony
EPO	erythropoietin
ET	essential thrombocythemia
FcγR	Fcγ receptor
FGF-2	fibroblast growth factor-2
FHL	four and a half LIM domain 1

FLP	ferritin light polypeptide
FLT-3	fms-like tyrosine kinase-3
FU	fluorescent unit
G6PD	glucose-6-phosphate dehydrogenase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
G-CSF	granulocyte colony stimulating factor
GEMM	granulocyte, erythroid, monocyte/macrophage, megakaryocyte
GM-CSF	granulocyte-macrophage colony stimulating factor
Gp-	glycoprotein
GVHD	graft verses host disease
HIT	heparin-induced thrombocytopenia
HPPC-MK	high proliferative potential cell megakaryocyte
HPP-CFU-MK	high proliferative potential colony forming unit
HT	hereditary thrombocytosis
HSC	haemopoietic stem cell
HU	hydroxyurea
HUMARA	human androgen receptor assay
IDS	iduronate-2-sulphotase
IFN-α	interferon-α
IgG	immunoglobulin G
IL-	interleukin
IL-6R	IL-6 receptor
ITAM	immune receptor tyrosine activation motif
JAK2	janus kinase 2
KD	Kawasaki disease
K-S	Kaposi sarcoma
LAP	latency associated protein
LIF	leukaemia inhibitory factor
LTBP	latent TGFβ binding proteins
M-CSF	macrophage-colony stimulating factor
MDS	myelodysplasia
MGDF	megakaryocyte growth and development factor
MK	megakaryocyte

MKb	megakaryoblast
mN1(2)	difference product 1 (2) – mRNA from haematologically normal control used as tester
MNC	mononuclear cell
MPLV	myeloproliferative acute leukaemogenic retrovirus
M_{rep}	monoclonal representation
nM1(2)	difference product 1 (2) – mRNA from ET patient with monoclonal myelopoiesis used as tester
NRD1	N-arginine dibasic convertase 1
N_{rep}	normal control representation
ORF	open reading frame
PB	peripheral blood
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PEG-rHuMGDF	pegylated recombinant human MGDF
PF4	platelet factor 4
PGK	phosphoglycerate kinase
pMKb	promegakaryoblast
proMK	promegakaryocytes
PV	polycythemia vera
PVSG	polycythemia vera study group
RANTES	regulated upon activation normal T cell expressed and secreted
RBC	red blood cell
RDA	representational difference analysis
$RF_{2\%}$	RPMI 1640 media with L-glutamine supplemented with 2% foetal calf serum
rhTPO	recombinant human thrombopoietin
RT	reverse transcription
sJCA	systemic juvenile chronic arthritis
SAGE	serial analysis of gene expression
SCF	stem cell factor
SLS	sample loading solution
SNP	single nucleotide polymorphism

SQ-RT-PCR	semi quantitative RT-PCR
SSCP	single strand conformational polymorphism
ssDNA	sheared single stranded DNA
STR	short tandem repeat
TCR	T-cell receptor
TGFβ (1)	transforming growth factor β (1)
TGFβR	TGFβ receptor
TPO	thrombopoietin
TSP	thrombospondin
UTR	untranslated region
VEGF	vascular endothelial growth factor
VNTR	variable number tandem repeat sequence
vWF	von Willebrand Factor
WBC	white blood cell
WHO	World Health Organisation
XCIP	X-chromosome inactivation pattern

Chapter 1

Introduction

1.1 Haemopoiesis

All blood cells in the circulation are produced by the proliferation and differentiation of a small pool of multipotent haemopoietic stem cells (HSC) primarily located in the bone marrow (BM). This process is termed haemopoiesis and is vital to the effective functioning of the circulatory system as it allows the many different types of specialist blood cells, each with a differing and finite life span, to be continuously replenished. The requirement for production of particular types of blood cell can vary dramatically, for example an acute bacterial infection requires a dramatic increase in neutrophil numbers, or red cell production needs to be increased in the event of blood loss, and the HSC/BM system is able to react to these stimuli in order to alter the rate of production of a specific haemopoietic cell when required. This balance occurs via regulation of cell proliferation, commitment, maturation, survival and removal. Mature haemopoietic cells are produced by proliferation of HSCs, whose progeny become progressively more committed to specific cell lineages. One of the first events appears to be commitment to either the myeloid or lymphoid cell lineages. Granulocytes, erythrocytes, monocytes/macrophages and megakaryocytes (MKs) form the myeloid lineage and so progenitor cells committed to the myeloid lineage are termed colony forming unit GEMM (CFU-GEMM). Early lymphoid committed progenitors are called CFU-L (Figure 1.1). These committed progenitor cells are limited in the number of cell types that they can produce, and as they proceed further along the differentiation pathway they mature until they become morphologically, immunologically and functionally distinct haemopoietic cells. Cell division also occurs during the maturation process which enables haemopoiesis to produce the large numbers of functionally distinct granulocytes (neutrophils, eosinophils, basophils), monocytes/macrophages, erythrocytes, platelets, T and B lymphocytes seen in the circulation.

Haemopoiesis is kept under tight control by a number of regulatory molecules that stimulate and coordinate proliferation of HSCs and their progeny, and which initiate progress along specific maturation pathways (Metcalf, 1998). These haemopoietic growth factors and cytokines are able to act on one or more lineages and often exhibit synergistic actions. Stem cell factor (SCF) and fms-like tyrosine kinase-3 (FLT-3) are key regulators of the initial generation of progenitor cells from HSCs. Simultaneous stimulation from several

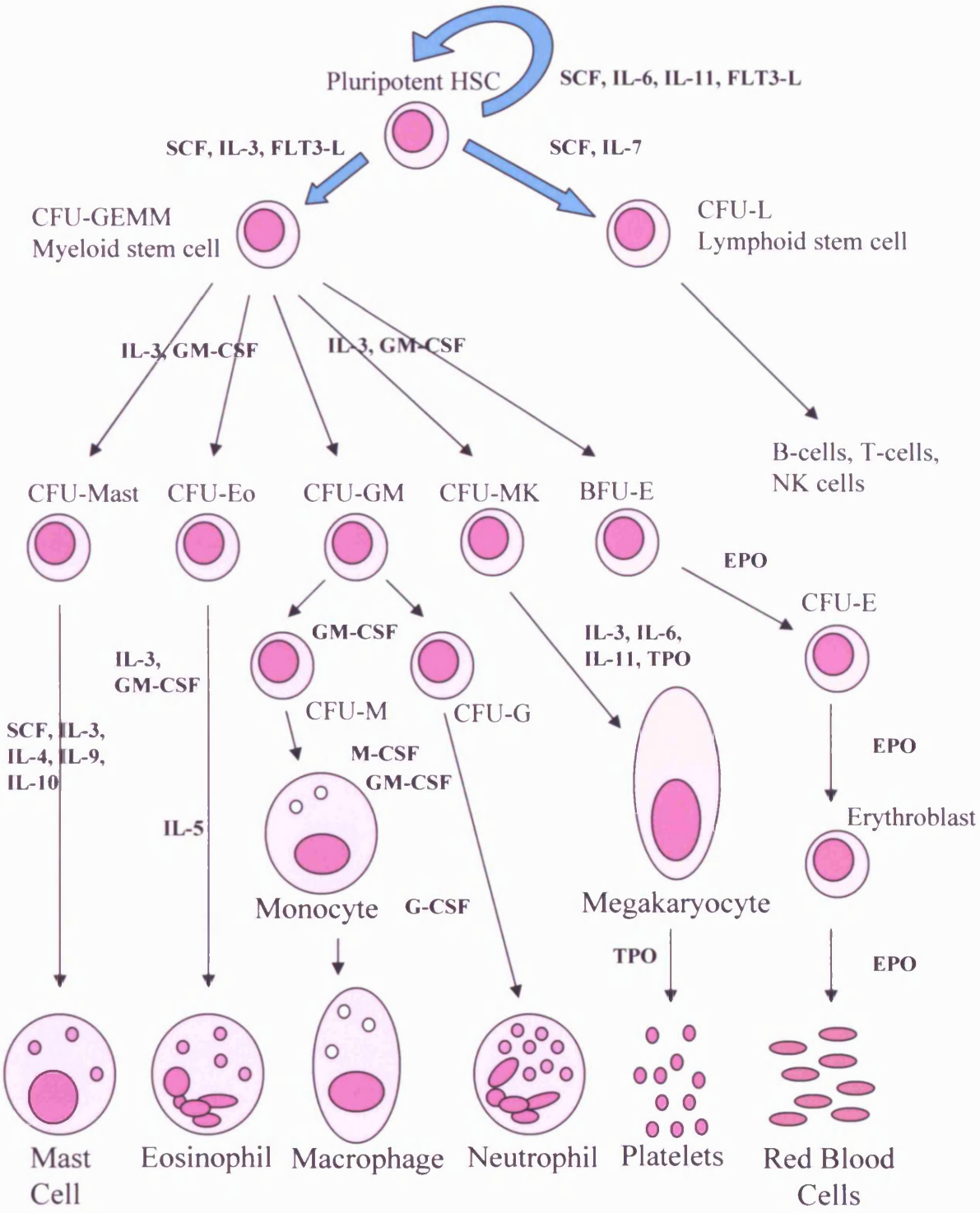

Figure 1.1

Model of haemopoiesis Haemopoietic stem cells (HSC) in the bone marrow differentiate into the various lineages under the control of cytokines and growth factors. The important cytokines and growth factors are shown in bold type. Adapted from Hara and Miyajima (1999)[†]. CFU – colony forming unit, BFU – burst forming unit, Eo – eosinophil, GM – granulocyte macrophage, MK – megakaryocyte, E – erythroid, NK – natural killer, SCF – stem cell factor, FLT3-L – fims like tyrosine kinase-3 ligand, IL- - interleukin, CSF – colony stimulating factor, TPO – thrombopoietin, EPO- erythropoietin.

† See additional references p255

regulators is required for the differentiation of stem cells, and it has been shown that the interleukins -3, -6, -7, -11 (IL-3, IL-6, IL-7, IL-11) and granulocyte and granulocyte-macrophage colony stimulating factors (G-CSF, GM-CSF) are important in SCF mediated stimulation. IL-3, G-CSF and thrombopoietin (TPO) are cytokines which are active in both the early and late stages of haemopoiesis. Factors such as G-CSF, GM-CSF, macrophage-colony stimulating factor (M-CSF) and IL-3 are important in the development of mature granulocytes and monocytes, and other regulators such as erythropoietin (EPO), G-CSF, M-CSF, and TPO have important actions in terms of commitment to a specific lineage. EPO is key for promoting the production of erythrocytes, G-CSF for neutrophils, M-CSF is specific for promoting monocyte and macrophage production and TPO is the prime regulator for platelet production (Fraser *et al*, 1996). A suitable microenvironment for the regulation of haemopoiesis is provided by BM stromal cells, which secrete regulatory growth factors and cytokines and allow cell-to-cell interactions to take place (Torok-Storb *et al*, 1999).

1.2 Thrombopoiesis

Thrombopoiesis is the process of proliferation and maturation of HSCs along the MK lineage to form mature platelet shedding MKs. The process can be artificially divided into 3 stages: a highly proliferative progenitor stage, an intermediate immature MK stage and a terminally differentiated mature MK stage (Long, 1998).

The earliest MK lineage restricted progenitor cell identified to date is the high proliferative potential cell MK (HPPC-MK) (Bruno *et al*, 1996). Isolated from foetal BM, the HPPC-MK has yet to be identified in adults, but shares the characteristics of the murine high proliferative potential colony forming unit MK (HPP-CFU-MK) isolated from both foetal and adult mice (Jackson *et al*, 1994a). Each HPPC-MK can generate a colony of 300-1000 MKs in semi solid culture medium after 21 days incubation in a serum-depleted fibrin clot assay system (Bruno *et al*, 1996). HPPC-MK express CD34 and CDw109 on their surface and mature into burst forming unit MKs (BFU-MK). BFU-MK are the earliest MK progenitor isolated from adult human BM and are able to produce colonies of 100-500 cells. While BFU-MK still express CD34, they have a more mature immunophenotype than HPPC-MK as they also express c-kit, the SCF receptor, at their cell surface. The most

mature cell in the progenitor stage of thrombopoiesis is the colony forming unit MK (CFU-MK), which gives rise to colonies containing 4-36 cells. CFU-MK are still CD34+ and c-kit+, but also express HLA-DR (Figure 1.2).

During the immature MK stage of thrombopoiesis progenitor cells mature morphologically and immunophenotypically and cease to proliferate. Two cell types make up the immature MK stage: the promegakaryoblast (pMKb) and the more mature megakaryoblast (MKb). pMKbs have restricted proliferative potential, but display mature MK markers at the cell surface such as the platelet glycoprotein GPIIb/IIIa (CD41) and von Willebrand factor (vWF), although they still express the immature marker CD34 (Rabellino *et al*, 1981). The endomitotic increase in DNA content characteristic of MKs mainly occurs in pMKbs (Ebbe & Stohlman, Jr., 1965), and they are the first cell in the MK lineage to increase in number during thrombocytosis (Jackson, 1973). MKbs are more mature than pMKbs, and it is during this stage of development that CD34 expression is lost. Morphologically, MKbs have a high nucleus to cytoplasm ratio and basophilic cytoplasm indicative of a high level of protein synthesis. MKbs have a very low proliferative potential even when positive for CD34 expression (Debili *et al*, 1992).

The mature MK stage of differentiation consists of promegakaryocytes (proMK) and fully mature, platelet shedding MKs. ProMK are CD34-, HLA-DR+ and CD41+. During this stage of maturation the nucleus to cytoplasm ratio increases further, and platelet specific granules start to appear. Mature platelet shedding MKs have an abundant mature cytoplasm and large lobulated nucleus. DNA content has increased by doublings to a model ploidy of 16N, although MKs with ploidy levels of 8-128N are normal. An increase in membrane synthesis occurs in mature MKs, which invaginates to form a tubular system at the cell margin termed the demarcation membrane system (DMS). The DMS is thought to divide the cytoplasm into platelet fields, although its exact role remains obscure. The final stage of thrombopoiesis occurs when long cytoplasmic projections extend from the MK periphery. Platelets detach from the tip of these projections and are released into the circulation (Cramer *et al*, 1997).

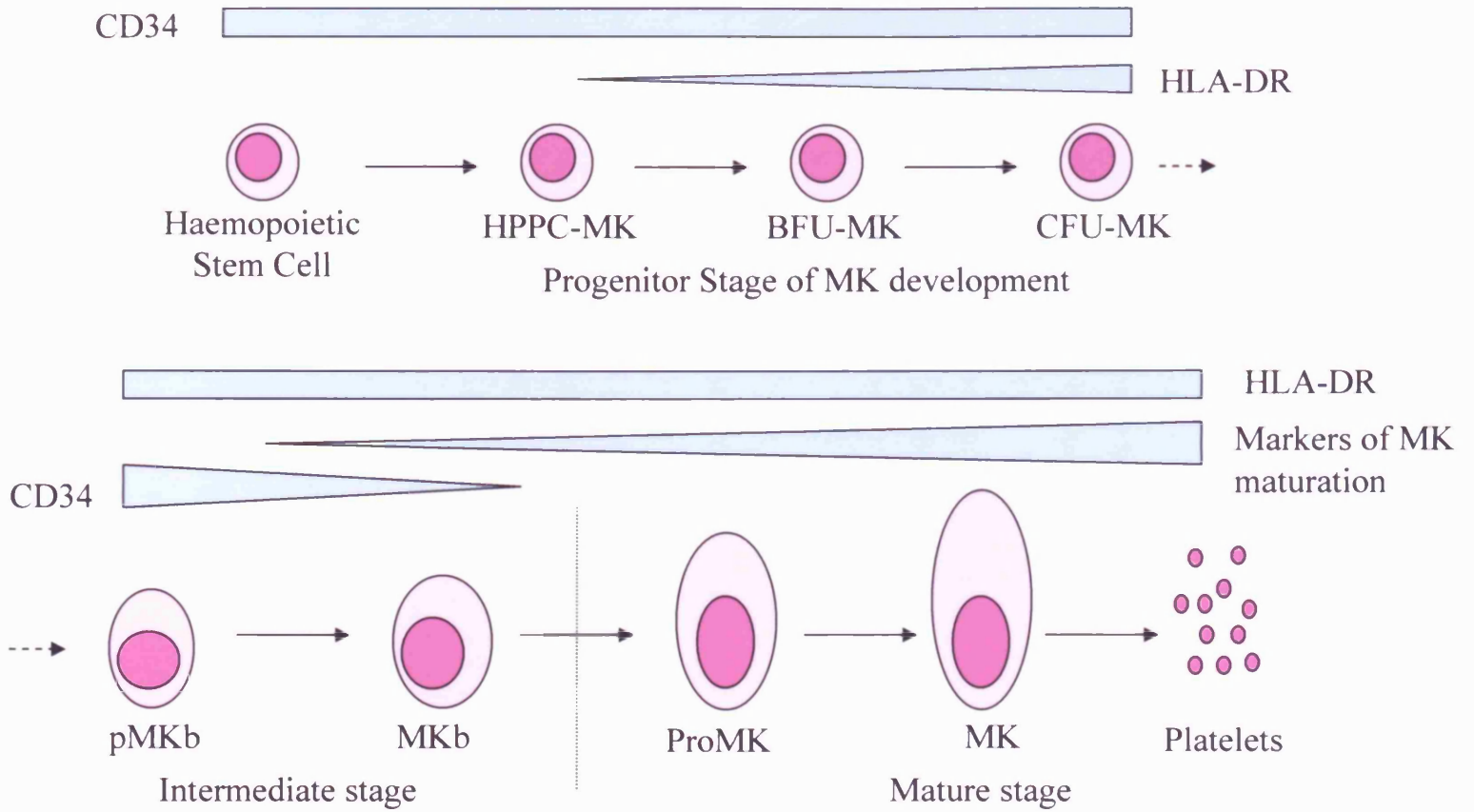

Figure 1.2

Overview of Thrombopoiesis

The cell types of the MK lineage are shown with the major cell surface markers present at each stage of maturation. HPPC-MK – high proliferative potential cell MK, BFU-MK – burst forming unit MK, CFU-MK – colony forming unit MK, pMKb – promegakaryoblast, MKb – megakaryoblast, ProMK – promegakaryocyte.

1.3 Regulation of Thrombopoiesis

1.3.1 Thrombopoietin and c-mpl

Thrombopoietin (TPO), also known as megakaryocyte growth and development factor (MGDF), is the prime regulator of thrombopoiesis and interacts specifically with its receptor Mpl on the surface of all cells of the MK lineage to promote proliferation, differentiation and ultimately platelet production. TPO was discovered in 1994 independently by four groups as the ligand to the then orphan receptor Mpl. It has since been shown to stimulate the proliferation and maturation of cells of the MK lineage at all stages of development and to be responsible for the increase in ploidy seen in MKs by stimulating endomitosis. However, TPO may have an inhibitory effect on the terminal phase of platelet formation, proplatelet budding, as it was shown that when MKs were plated in the presence of a number of cytokines, TPO specifically reduced platelet formation (Ito *et al*, 1996).

1.3.2 Stem Cell Factor

SCF on its own has little effect on thrombopoiesis. However, it acts synergistically with TPO, IL-6, IL-3 and GM-CSF to stimulate thrombopoiesis. This was demonstrated with IL-3 and GM-CSF by an increase in cloning efficiency and size of MK colonies in clonogenic assays with the addition of SCF (Briddell *et al*, 1991). Also, TPO activation of STAT5 was enhanced 1.6 fold in Mo7e cells by costimulation with SCF (Drayer *et al*, 2005). Platelet counts in mice treated with SCF have been shown to increase by 30% (Chow *et al*, 1993). However, this was not reproducible in primate models where BM MK levels increased but not platelet counts in the peripheral blood (Andrews *et al*, 1994). SCF may also have an effect on mature MKs, inducing polyploidisation and enhancing the terminal stages of maturation (Avraham, 1993; Debili *et al*, 1993). SCF-deficient mice have abnormal thrombocytosis, with a reduction in the number of MKs and an increase in MK size (Hunt *et al*, 1992).

1.3.2 IL-3 / GM-CSF

GM-CSF and IL-3 are functionally related glycoproteins which both stimulate MK colony growth, although IL-3 is more potent and can act directly, whereas GM-CSF

appears to act in synergy with IL-3 to promote thrombopoiesis. In serum-free cultures IL-3 seems to act on the late stages of MK differentiation (Suda *et al*, 1986). Administration to mice augments platelet production (Carrington *et al*, 1991) and clinical trials have shown that this is also the case in humans. A hybrid IL-3/GM-CSF molecule PIXY321 was shown to support various stages of thrombopoiesis *in vitro* and in a phase I study was found to be effective at increasing both neutrophil and platelet counts in patients with sarcoma (Vadhan-Raj *et al*, 1994). However, phase III trials have shown that PIXY321 is no better than GM-CSF alone in supporting thrombopoiesis (O'Shaughnessy *et al*, 1996) and a further study showed no effect on thrombopoiesis but systemic toxicity, so the clinical impact of PIXY321 has been small (Jones *et al*, 1999).

1.3.3 IL-6, IL-11 and Leukaemia inhibitory factor

IL-6, IL-11 and leukaemia inhibitory factor (LIF) are members of a family of cytokines which have similar, but not identical, modes of action on thrombopoiesis. IL-6 supports MK maturation in the absence of other growth factors and can augment platelet production in addition and synergy with other growth factors. However, IL-6 knock-out mice have normal platelet counts so it is unlikely IL-6 is crucial for MK development (IL-6 is discussed in more detail in Chapter 6 – 6.1.5). IL-11 is similar in its actions to LIF, inducing MK progenitor maturation and proliferation. However, IL-11 can also act alone to promote MK maturation (Bruno *et al*, 1991a). When administered to mice, IL-11 increases the number of MK progenitors, stimulates polyploidisation of BM MK and increases platelet count (Neben *et al*, 1993). However, mice deficient for IL-11 have normal levels of circulating platelets (Gainsford *et al*, 2000). Mice deficient for LIF do not develop thrombocytopenia but they do have dramatically reduced numbers of progenitor cells in their BM and spleen (Escary *et al*, 1993).

1.3.4 Erythropoietin and other cytokines

Erythropoietin (EPO)-enriched preparations were a requirement in early MK culture systems (Vainchenker *et al*, 1979), and it has been suggested that EPO exerts its action mainly in the maturation stages of MK development. The EPO receptor is found on MKs, and clinical administration of EPO can lead to an increase in platelet count (Stone *et al*, 1988). However, EPO is unlikely to play a significant role in thrombopoiesis as patients with chronic renal failure who are treated with EPO show no increase in platelet counts,

and anephric animals and patients, made anaemic by reduced EPO expression, do not display thrombocytopenia. IL-1 has no direct effect on thrombopoiesis, but it does induce the synthesis of factors that do, such as SCF, IL-6 and GM-CSF. IL-1 can also act in synergy with TPO and IL-6 to promote MK proliferation. IL-10 has been shown to stimulate the proliferation of CFU-MK, but only in combination with EPO, IL-3, or IL-11. IL-13 continuously administered to mice can increase the numbers of immature MKs in the spleen but this does not lead to significant changes in platelet numbers. The major cytokines involved in thrombopoiesis and the stage of the lineage at which they influence development is outlined in Figure 1.3.

1.4 Chronic Myeloproliferative disorders

Essential thrombocythaemia (ET) is one of a group of disorders termed chronic myeloproliferative disorders (cMPD) which are characterised by dysregulation of myeloid elements. The term cMPD was first used by Damashek in 1951 who suggested that these interrelated disorders arose from a defect in the BM causing hyperproliferation of cells of the myeloid lineage. The cMPDs consist of four major disorders, chronic myeloid leukaemia (CML), chronic idiopathic myelofibrosis (cIMF), polycythaemia vera (PV) and ET, although CML is often considered separately from the others as its clinical course is more aggressive and its aetiology is better understood. CML patients are characterised by a t(9;22) translocation which produces the fusion protein BCR-ABL. The chromosome produced by the translocation is called the Philadelphia chromosome (Ph^1). Non-CML cMPDs are often referred to as the Ph^1 negative cMPDs. The Ph^1 negative cMPDs are classified according to predominant phenotype. As the underlying defect involves a common haemopoietic progenitor, categorisation can be difficult, for example all cMPDs may have an associated thrombocytosis, but in each disorder the proliferation of one cell type predominates, platelets in ET, red cells in PV and fibroblasts in cIMF. A cMPD can acquire features of another cMPD and therefore patients may switch to another cMPD during the course of the disease. However, the clinical course and management differs between each syndrome so a correct diagnosis is essential.

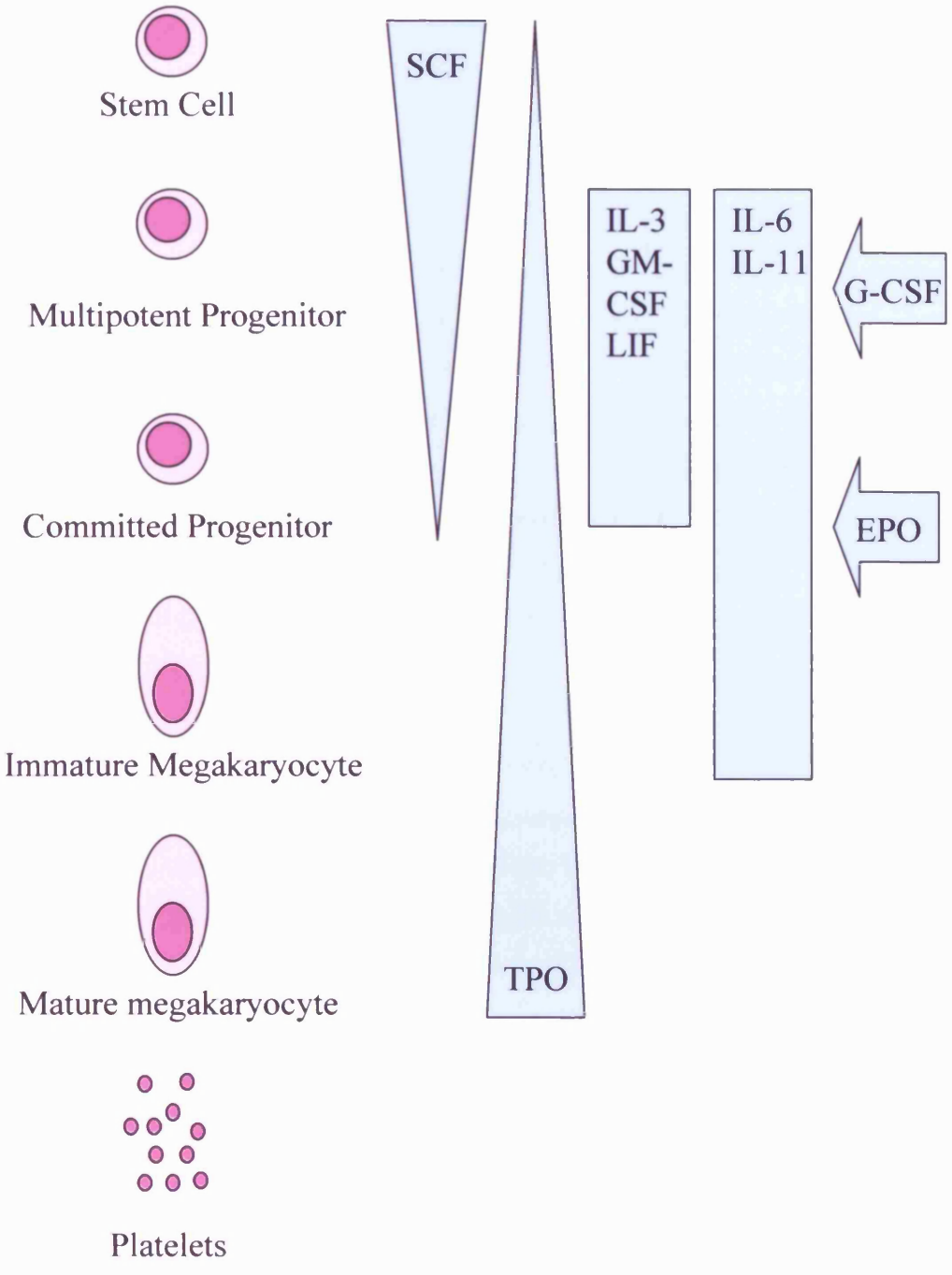

Figure 1.3 Cytokine involvement in thrombopoiesis

1.5 Essential Thrombocythaemia

1.5.1 Overview

ET is characterised by a sustained thrombocytosis of the blood and megakaryocytosis of the BM. A blood smear may display abnormal platelets, giant platelets are often present, and abnormally shaped platelets are sometimes, but not always, observed. In the BM a marked proliferation of large, mature MKs with multi-lobed nuclei is characteristic. Although the clinical course of ET is often benign, the main complications for patients are life threatening thrombotic and/or haemorrhagic events. The aetiology of ET is unknown and no disease-specific markers have been identified to date. Therefore, diagnosis of ET is currently one of exclusion of other causes of thrombocytosis which include inflammation and infection, solid tumours and other cMPDs. ET is estimated to occur in 1-2.5/100,000 of the population, although the true incidence is unknown (Mesa *et al*, 1999). The modal age at diagnosis is 50-60 years, and there is no predilection for either sex. However, a second diagnostic peak is seen at 30 years where women are more frequently affected (Mesa *et al*, 1999; Murphy *et al*, 1986).

1.5.2 Diagnosis

The diagnostic criteria for ET was first established by the polycythemia vera study group (PVSG) in 1976. The criteria contained guidelines for the exclusion of other cMPDs and a diagnostic platelet threshold of 1000 x 10^6/ml. This criteria was subsequently updated in 1986 to reduce the diagnostic platelet threshold to 600 x 10^6/ml (Murphy *et al*, 1986). The criteria was again modified in the final report of the PVSG in 1997 to include a clause to exclude myelodysplasia (Murphy *et al*, 1997). Thus a diagnosis of ET by the PVSG criteria requires a platelet count >600 x 10^6/ml, PV exclusion by either haematocrit of less than 40 or a normal red cell mass in the presence of normal iron stores, exclusion of myelofibrosis by the absence of collagen fibrosis (less than 33% fibrosis of trephine biopsy) with a lack of leucoerythroblastic features on a blood film and exclusion of CML by lack of Ph^1. More recently these criteria have been updated by the World Health Organisation (WHO) to including BM morphology in the diagnostic criteria, and its description of ET is outlined below (Pierre *et al*, 2001). The PSVG and WHO diagnostic

criteria are given in Table 1.1 and Table 1.2 respectively. Most studies into the biological and/or molecular pathogenesis of ET have used the PVSG criteria to diagnose patients.

1.5.3 Clinical Features

More than 50% of all ET patients are asymptomatic at diagnosis, and are often identified from a routine blood count showing an increase in platelet numbers. However, 20-50% of patients present with a vascular occlusion or haemorrhage. (Pierre *et al*, 2001) Microvascular occlusion can occur, often leading to cerebral or digital ischaemia, as well as thrombosis of the major arteries and veins. Haemorrhage, commonly from the mucosal surfaces of the upper airway passages and gastrointestinal tract has also been documented. Approximately 50% of ET patients display modest splenomegaly and 15-20% hepatomegaly. (Pierre *et al*, 2001) (Murphy *et al*, 1997)

1.5.4 Cell morphology

A marked thrombocytosis is the most striking feature of a blood smear obtained from an ET patient. The platelets are often unequal in size, ranging from tiny forms to giant platelets, and while unusually shaped platelets can occur, they are uncommon. The numbers of white blood cells (WBCs) in the peripheral blood (PB) are often mildly elevated. Red blood cells (RBCs) are normal except where haemorrhage has occurred, when the RBCs often show signs of iron deficiency. (Pierre *et al*, 2001)

BM aspirate smears show a large increase in MK numbers compared to normal with large sheets of platelets also evident. Stainable iron is present in 40-70% of patients at diagnosis. (Pierre *et al*, 2001)

BM trephines are often normal in appearance but for a marked megakaryocytosis, with large to giant MKs. The MKs are usually found to be in loose clusters, but can be regularly dispersed throughout the BM, and have deeply lobulated and hyperlobulated nuclei. The presence of a normal or minimally increased reticulin fibre network is characteristic of ET, a large increase in the amount of reticulin fibres in the BM biopsy is indicative of the early stages of cIMF. (Pierre *et al*, 2001)

Extramedullary tissues are rarely involved in the MK proliferation, and where extramedullary haemopoiesis is seen in the liver or spleen, it is usually scant. The spleen may become enlarged due to high levels of platelets and platelet aggregates filling the splenic sinuses and cords. However, this often does not occur because of splenic atrophy due to platelet 'clogs'. (Pierre *et al*, 2001)

1 Platelet count >600x106/ml

2 Haematocrit <40 or normal red cell mass(males <36ml/kg, females <32ml/kg)

3 Stainable iron in the marrow or normal RBC mean corpuscular volume*

4 No Philadelphia chromosome or *bcr/abl* gene rearrangement

5 Collagen fibrosis of the bone marrow

A: Absent or

B: Less than one third of the biopsy area without both marked splenomegaly and a leucoerythroblastic blood film

6 No cytogenetic or morphological evidence for myelodysplastic syndrome

7 No cause for reactive thrombocytosis.

Table 1.1
Polycythaemia vera study group criteria for the diagnosis of ET.
* If these measurements suggest iron deficiency, PV cannot be excluded unless a trial of iron therapy fails to increase the red cell mass into the polycythaemic range.

Positive Criteria

1 Sustained platelet count ≥600 x 10^6/ml

2 Bone marrow biopsy specimen showing proliferation mainly of the MK lineage with increased numbers of enlarged, mature megakaryocytes

Criteria of exclusion

1 No evidence of polycythaemia vera (PV):
- Normal red cell mass Hb <18.5g/dl in men, 16.5g/dl in woman
- Stainable iron in marrow, normal serum ferritin or normal MCV
- If the former condition is met, failure of iron trial to increase red cell mass or Hb levels in the PV range

2 No evidence of CML:
- No Philadelphia chromosome and no BCR/ABL fusion gene

3 No evidence of chronic idiopathic myelofibrosis:
- Collagen fibrosis absent
- Reticulin fibrosis minimal or absent

4 No evidence of myelodysplastic syndrome:
- No del(5q), t(3;3)(q21;q26), inv(3)(q21;q26)
- No significant granulocytic dysplasia, few if any micromegakaryocytes

5 No evidence that thrombocytosis is reactive due to:
- Underlying inflammation or infection
- Underlying neoplasm
- Prior splenectomy

Table 1.2
World health organisation diagnostic criteria for essential thrombocythaemia

1.5.5 Cytogenetics

To date, no specific cytogenetic marker has been identified for ET, although a small number of patients do have cytogenetic abnormalities. Cytogenetic studies need to be carried out routinely in ET patients though, as exclusion of the Ph^1 chromosome is essential for exclusion of CML at diagnosis, therefore it is unlikely that a common gross chromosomal abnormality would have been overlooked. Approximately 5-10% of ET patients do have chromosomal abnormalities, the most commonly occurring are del(13q22), +8 and +9. (Pierre *et al*, 2001) (Barbui *et al*, 1999)

Many distinctive abnormalities are associated with rearrangements of chromosome 17, particularly in patients that have progressed to myelodysplasia (MDS) or acute myeloid leukaemia (AML). However, the possibility that these alterations are therapy-related is high as most of these patients were treated with hydroxyurea (HU) prior to transformation. (Finnazi *et al*, 1999) (Murphy *et al*, 1997)

1.5.6 Prognosis

ET is an indolent disorder, with long periods of symptom-free intervals, punctuated with life-threatening thromboembolic or haemorrhagic events. Median survival times of 10-15 years are common, and because modal diagnosis of ET is at 50-60 years of age, many patients have a near normal life expectancy (Tefferi, 2001) †. Splenectomy can result in an increase in platelet count and increased mortality as the spleen is a major sequestration site for platelets. ET can transform into other less benign cMPDs such as PV or cIMF but this is only an occasional occurrence. Fewer than 5% of patients transform to AML or a myelodysplastic syndrome, although it is difficult to determine whether this is due to genuine disease progression or is related to cytoreductive therapy. (Tefferi *et al*, 2000) †

The major complication in ET is thrombosis, in one study of 100 patients haemorrhagic events occurred at the rate of 0.33%/patient-year (pt-yr), but thrombotic events were noted in 6.6%/pt-yr (Cortelazzo *et al*, 1990). The rate of thrombosis could be matched to patient age and previous history of thrombosis. Patients less than 40 years of age had a thrombotic rate of 1.7%/pt-yr, 40-60 years of age 6.3%/pt-yr and patients aged over 60 years 15.1%/pt-yr. In patients with a previous history of thrombosis the thrombotic rate increased from 3.4-31.4/pt-yr, even though all these patients were receiving treatment with cytoreductive therapy. In the light of these figures, ET patients can be classed as low risk if they are asymptomatic and less than 60 years of age, or high risk if they have a history of thrombosis or are aged over 60 years. A platelet count in excess of 1500 x 10^6/ml

† See additional references p255

would also signify high risk as an increase in the risk of haemorrhage has been noted in these patients (van Gameren *et al*, 1994). However, increasing platelet count does not correlate with an increase in thrombotic events.

1.5.7 Therapy

A number of therapeutic strategies are available to control thrombocytosis in ET. In low risk patients, withholding chemotherapy is thought to be the best therapy, although anti-aggregation therapy such as aspirin is often still used. In a study of 65 ET patients under 60 years of age with no history of thrombosis or major bleeding and a platelet count of less than 1500 x 10^6/ml, cytoreductive therapy was withheld until occurrence of a major clinical event (Cortelazzo *et al*, 1990). After a median 4.1 year follow-up, the incidence of thrombosis was 1.91%/pt-yr for ET patients and 1.43%/pt-yr for sex matched normal controls. No major bleeding was observed. This study indicated that low risk ET patients are not at higher risk of thrombotic events than the normal population. These findings were supported by another study which followed 28 low risk ET patients for a median of 4.6 years and found that only 1 thrombotic event occurred during the time of the study (Randi *et al*, 1999).

In high risk patients, cytoreductive therapy is recommended. However, there are a number of different agents that can be administered, and each has its own drawbacks.

Hydroxyurea

HU is non-alkylating antimetabolite which inhibits the enzyme ribonucleoside reductase, preventing DNA synthesis. The convenience of use, efficacy and low acute toxicity of HU has made it a common treatment choice in ET. The dose administered can be adjusted to maintain maximal platelet reduction without excessive lowering of WBC count, and continuous treatment reduces platelet counts to below 500 x 10^6/ml in 80% of patients within 8 weeks of the initial dose (Barbui *et al*, 1999). The major short term toxic effect is leukopenia caused by haemopoietic damage, but this is reversed upon withdrawal of HU. Discontinuation of HU is accompanied by a rebound of platelet count, so continuous administration is necessary. Patients can become resistant to the effects of HU and may also develop serious side effects which limits its usefulness in some individuals. (Barbui *et al*, 1996)

Significant improvements in reducing the occurrence of thrombotic events can be achieved with the use of HU. A trial involving 114 ET patients with median age 68 years

(range 40-85), and median platelet count at entry to trial 780 x 10^6/ml (range 533-1240) was carried out over 27 months (Cortelazzo *et al*, 1995). HU was administered to 56 patients, the other 58 patients received no cytoreductive therapy. In the group receiving HU, only 2 thrombotic events were recorded (1.6%/pt-yr), but in the group receiving no cytoreductive therapy there were 14 thrombotic events (10.7%/pt-yr) (p=0.003). After 78 months follow-up, 5 patients from the HU group had a thrombotic event compared to 26 from the non-HU treated group (Finazzi *et al*, 2000).

As a non-alkylating agent, HU was originally thought to be non mutagenic. However, long term studies of patients treated with HU have shown 5-10% develop acute leukemia (Sterkers *et al*, 1998). More recently, trial data has shown that while none of the 20 ET patients receiving no cytoreductive therapy developed secondary malignancies, only 3 out of 77 patients receiving HU alone did, but 5/15 patients who received HU after previously receiving busulphan developed malignancies (Finazzi *et al*, 2000). This suggests that the rate of transformation to AML or a myelodysplastic syndrome with HU alone is minor, but that the combination of HU and other chemotherapeutic agents may significantly increase the risk of secondary malignancies.

Interferon-α (IFN-α)

IFN-α is a naturally occurring agent with antiproliferative actions in haemopoiesis and is a myelosuppressive. The response rate for platelet reduction with the administration of IFN-α is high at approximately 90% (Barbui *et al*, 2005). During maintenance the dose can be altered to tightly regulate platelet counts, but if treatment is suspended platelet counts rebound. Long term use of IFN-α is severely limited by side effects; in a review of 273 patients published in the literature, 206 cases (75%) were either withdrawn from studies due to side effects (55%) or patients refused to comply with treatment regimens or were lost to follow-up (20%) (Lengfelder *et al*, 1996).

Anagrelide

Anagrelide is an oral imidazo-quinazoline derivative which specifically suppresses platelet production. Although its mode of action is poorly understood, it is thought to interfere with MK maturation. The efficacy of anagrelide as a treatment for ET has been assessed in non-comparative clinical studies. A response was defined as a reduction in platelet count to less than 500 or 600 x 10^6/ml, or a 50% drop in platelet count, and a

response rate of 60-93% has been described in a total of 577 patients (Anagrelide Study Group, 1992). However, neurological and gastrointestinal complications have been reported, as well as two sudden deaths related to heart disease. More recently a study of 37 young ET patients (median age 40 years, range 18-49) with a median follow-up of 10.7 years (range 5.2-13.7 years) showed that while the response rate was 90%, and the reduction in platelet count was sustained in over 80% of patients, 4 patients (11%) discontinued treatment due to toxicity, thromboembolic complications occurred in 7 patients (19%) and haemorrhagic complications in 6 patients (16%). None of these patients developed a secondary malignancy (Storen & Tefferi, 2001). Also, the PT-1 trial investigating 809 ET patients randomized to receive either HU and aspirin or anagrelide and aspirin has shown that anagrelide and aspirin use was associated with an increase risk of arterial thrombosis, haemorrhage and transformation to cIMF compared to HU and aspirin therapy (Green *et al*, 2004). Therefore, while anagrelide is well tolerated in most patients and efficient at lowering platelet counts, it may not prevent thrombotic or haemorrhagic events with long term use.

1.6 Potential Molecular Markers of ET

1.6.1 Clonality

Chromosomal alterations are rare in ET and therefore studies to determine clonality status in patients have predominantly relied on an indirect technique which analyses the relative expression of X-linked alleles in females – i.e. the X-chromosome inactivation pattern (XCIP). Initial studies using XCIPs suggested that ET was a clonal disorder (Anger *et al*, 1990; Fialkow *et al*, 1981; Raskind *et al*, 1985), but advances in the biologic and technical understanding of this technique have indicated a need for these results to be reappraised. This subject is discussed further in chapters 3 and 4, but an overview is given below.

A clonal XCIP pattern has been arbitrarily defined as 75% expression of one allele, but approximately 25% of normal females have a constitutively imbalanced pattern probably due to the small number of HSCs present at the time of X-inactivation. Therefore T-cells are analyzed to control for this (Gale *et al*, 1991). Also, the myeloid lineage is more

susceptible to acquired age-related skewing of XCIPs than the T-cell lineage and therefore only patients less than 65 years of age are defined as clonal by XCIP (Gale *et al*, 1997). Using these caveats, and larger numbers of patients, it has been shown that a large proportion of ET patients have polyclonal myelopoiesis, ranging from 31% (Shih *et al*, 2002) to 57% (Harrison *et al*, 1999a) with a mean of 40% from all studies (Chiusolo *et al*, 2001; el Kassar *et al*, 1997; Ferraris *et al*, 1999; Liu *et al*, 2003; Shih *et al*, 2002; Teofili *et al*, 2002a; Vannucchi *et al*, 2004). Four groups have independently demonstrated an association between the presence of clonal myelopoiesis and increased risk of thrombosis, suggesting that ET patients could be divided into two groups, one having polyclonal myelopoiesis and a second with clonal myelopoiesis and an increased risk of thrombotic events (Chiusolo *et al*, 2001; Harrison *et al*, 1999a; Shih *et al*, 2002; Vannucchi *et al*, 2004).

1.6.2 Thrombopoietin and Mpl

TPO is the primary physiological regulator of thrombopoiesis, and as such much research has been carried out to identify abnormalities in this cytokine in ET patients. This subject is discussed in more detail in chapter 5, but an overview is given below.

Studies on the inherited form of ET, hereditary thrombocythaemia (HT), have shown that mutations in the 5' untranslated region (UTR) of the TPO gene can lead to an increase in TPO expression (Ghilardi *et al*, 1999; Jorgensen *et al*, 1998; Kondo *et al*, 1998; Wiestner *et al*, 1998). However, these mutations have not yet been shown to be present in ET patients (Harrison *et al*, 1998b).

Normal or elevated serum or plasma TPO concentrations have been widely reported in ET patients (el Kassar *et al*, 1998; Griesshammer *et al*, 1998; Harrison *et al*, 1999c). This finding is counter-intuitive as TPO is thought to be constitutively produced and circulating levels are mainly regulated by, and inversely proportional to, platelet and MK mass (Kuter & Rosenberg, 1995). The evidence seems to suggest that this may be due to reduced clearance rather than a defect in TPO expression. TPO is removed from the circulation by binding its specific receptor Mpl on the surface of MKs and platelets. A number of studies have demonstrated that mRNA expression of the Mpl gene, c-mpl, is reduced in ET and that the surface expression of Mpl protein is also reduced (Harrison *et al*, 1999c; Horikawa *et al*, 1997; Li *et al*, 2000). This would prevent the removal of TPO from the circulation, leading to the normal or elevated levels of circulating TPO seen in

patients. However, elevated TPO levels are also seen in other cMPDs and in patients with reactive thrombocytosis (Harrison *et al*, 1999c), and platelet Mpl levels do not correlate with thrombosis (Vannucchi *et al*, 2004).

Normal BM cells stained for Mpl have a strong uniform staining pattern, but ET patients have a characteristically heterogeneous pattern of staining, with variation of both the site (membranous or cytoplasmic) and intensity (some strong, but predominantly moderate or weak) of staining. Also, not all cells are Mpl positive (Harrison *et al*, 1999c; Mesa *et al*, 2002).

The expression of TPO may not be entirely constitutive, IL-6 has been shown to augment TPO production in mice in a dose-dependent manner, which led to a 2.5 fold increase in platelet count (Kaser *et al*, 2001). Also, addition of the platelet α-granule proteins, platelet derived growth factor (PDGF) and fibroblast growth factor-2 (FGF-2), led to increased TPO mRNA expression in cultures of normal BM stromal cells by 2 and 1.5 fold respectively (Sungaran *et al*, 2000). Transforming growth factor β 1 (TGFβ1) added to cultured BM stromal cells from normal individuals also led to elevated TPO mRNA expression, up to 7 times normal levels (Sakamaki *et al*, 1999). This effect could be blocked by addition of a TGFβ blocking antibody prior to treatment, showing that the effect was due to TGFβ1 alone. Thus the normal or raised expression of TPO could be due to a combination of both decreased clearance and increased expression due to stimulation by other cytokines.

1.6.3 PRV-1 expression

A novel cell surface receptor, termed PRV-1, has recently been cloned using subtractive hybridisation techniques (Temerinac *et al*, 2000). It is most closely related to the uPAR/Ly6/CD59 receptor and its expression appears to be restricted to haemopoietic cells. Initial studies found PRV-1 to be overexpressed in granulocytes obtained from PV patients at the mRNA level. The initial study used Northern blot analysis to demonstrate PRV-1 mRNA expression in all of 19 PV patients studied and 4 out of 6 ET patients, but not in the 21 normal controls or the 4 patients with secondary erythrocytoses (Temerinac *et al*, 2000). Further studies by this group using a more sensitive quantitative RT-PCR method found that 18 out of 29 ET patients studied had a raised level of PRV-1 mRNA compared to normal controls, as did all the 48 PV patients, and that those ET patients with raised

PRV-1 were also positive for endogenous erythroid colony (EEC) growth, suggesting masked PV (Klippel *et al*, 2001). One study, using a non-quantitative method, has shown that all of 37 ET patients had raised PRV-1 levels compared to 20 normal control samples, but 25 patients with reactive thrombocytosis did not (Teofili *et al*, 2002a). However, others have not reproduced these results and most studies show a proportion of the ET patients with high levels of PRV-1 expression, but with the rest having normal levels. The proportion of ET patients with high PRV-1 mRNA expression ranges from 16% of 12 patients studied to 100% (Liu *et al*, 2003; Florensa *et al*, 2004; Tefferi *et al*, 2004; Teofili *et al*, 2002a). One group has been able to show an association between PRV-1 positivity, EEC growth and the risk of thrombotic events. In a cohort of 30 ET patients 15 (50%) had increased expression of PRV-1 and EEC growth and 12 of those 15 (80%) patients had a thromboembolic or microcirculatory complication, compared to 4/15 (26%) PRV-1 negative patients (Griesshammer *et al*, 2004). However, a second group could not demonstrate a correlation between PRV-1 expression and either thrombosis or clonality in 88 ET patients, suggesting that while PRV-1 may be useful as a marker for diagnosis of cMPDs, it may not have clinical relevance (Vannucchi *et al*, 2004). High PRV-1 levels may just reflect hyper-activation present in these disorders. Granulocytes from normal individuals treated with G-CSF to stimulate proliferation of the myeloid lineage also have high levels of PRV-1 mRNA expression, as do mature granulocytes stimulated with either G-CSF or GM-CSF *in vitro* (Temerinac *et al*, 2000), suggesting that highly proliferating myeloid cells may increase PRV-1 expression.

1.6.4 Endogenous Erythroid and MK Colony formation

Erythroid progenitor cells isolated from peripheral blood or BM of PV patients have the ability to proliferate and differentiate in the absence of the major cytokine of the erythroid lineage, EPO (Correa *et al*, 1994; Prchal & Axelrad, 1974). This process is termed endogenous erythroid colony (EEC) formation and is considered diagnostic in PV. However, approximately 50% of ET patients also form EECs. It has been suggested that these patients have a masked PV, but whether this is the case is unclear as the number of ET patients who transform to PV is low (Shih & Lee, 1994). In a recent study of 88 ET patients, 39.4% were positive for EECs, but no association could be shown between rates of thrombosis and EEC positivity (Vannucchi *et al*, 2004). This suggests that while EEC positivity may be a useful marker to aid identification of cMPD from reactive disorders, it

does not contribute to thrombotic risk. Assays for EECs are notoriously difficult to standardise due to technical variations in the procedure and the inherent variation between different batches of foetal calf serum and media. They are also time consuming to conduct and deciding if an assay is positive is highly subjective, therefore assays for EEC are not currently used widely for cMPD diagnosis.

MK progenitor cells isolated from the BM or peripheral blood of ET patients may also have the ability for spontaneous colony formation in the absence of the main regulator of the MK lineage TPO. Some studies have found that endogenous MK colonies are detectable and can be used to discriminate ET from a reactive disorder. In a culture system using 30% plasma from haemochromatosis patients, BM samples from all 24 patients with ET and 18/36 patients with other cMPDs grew spontaneous MK colonies, but such colonies were absent in cultures from reactive patients or normal control samples (Rolovic *et al*, 1995). However, other groups have not been able to reproduce these data and have found that not all ET patients grow spontaneous MK colonies (Abgrall *et al*, 1992; Juvonen *et al*, 1993), and some even describe colonies in cultures from patients with reactive thrombocytosis (Hamaguchi *et al*, 1988). More recently a report described the *in vitro* hypersensitivity of peripheral blood mononuclear cells from ET patients to TPO (Axelrad *et al*, 2000). However, as only 18/26 ET patients displayed hypersensitivity, and this was also observed in 1/8 patients with a reactive thrombocytosis, it would not be possible to use this test to confirm a diagnosis of ET.

1.6.5 Other markers

EPO A significant proportion of ET patients have reduced levels of circulating EPO measured from both serum and plasma samples. A study of serum samples from 49 ET patients showed that 16 (33%) had subnormal EPO levels by ELISA, and there was an association between these patients and an increased risk of thrombosis (Messinezy *et al*, 2002). A similar study of plasma samples obtained from 65 ET patients demonstrated that approximately 50% of the patients had low EPO using an immunoradiometric method (Andreasson *et al*, 2000). However, patients treated with cytoreductive therapy do not have subnormal EPO, one study reported that 12 out of 24 plasma samples from untreated ET patients obtained at diagnosis had subnormal EPO, but none of the 28 samples from patients that had received cytoreductive treatment were outside of the normal range (Carneskog *et al*, 1998). This effect is also seen in serum; in samples obtained from

untreated ET patients a mean EPO concentration of 6.6 mU/ml ± 8.0 (range 4.5-26.1) was recorded, compared to mean levels of 9.4 mU/ml ± 3.7 (range 2 – 17.9) for normal control samples and 14.1 ± 8.0 (range 4.5 – 26.1) for patients receiving cytoreductive therapy (Griesshammer *et al*, 2000). Also, EPO levels in plasma samples from all 20 ET patients analysed before and after treatment in one study rose to normal levels after treatment (Andreasson *et al*, 2000). However, the significance of this is still unclear as subnormal EPO levels do not correlate with clonality status (Andreasson *et al*, 2003) and have not yet been definitively shown to differentiate patients with higher risk of thrombosis.

NF-E2 and Antiphospholipid antibodies The transcription factor NF-E2 is involved in MK maturation and platelet production, independent of the actions of TPO (Shivdasani *et al*, 1995). In $CD34^+$-derived MKs obtained from 5 normal controls and 8 ET patients, the level of NF-E2 in ET patients was decreased at the mRNA level. The levels of 2 isoforms were measured, the a and f isoforms. Both isoforms were significantly reduced when compared to expression levels of GAPDH control gene by quantitative-RT-PCR, the ratio of NF-E2/GAPDH for the a isoform was 0.32 (range 0.24-0.4) in controls and 0.085 (range 0.026-0.16) in ET samples, and the ratio for the f isoform was 0.03 (range 0.025-0.042) in controls and 0.01 (range 0.006-0.027) in ET samples. This was shown to lead to a reduction in expression of the thromboxane synthase gene, a gene which is directly regulated by NF-E2. However, no decrease in protein expression could be demonstrated in the a isoform, but it could be demonstrated in the f isoform. It was suggested that this dysregulation of NF-E2 production may alter activity rather than absolute levels of NF-E2 in some ET patients. (Catani *et al*, 2002) †

Antiphospholipid antibodies (APA) have been shown to be risk factors for thrombosis in primary antiphospholipid syndrome and systemic lupus erythematosis (Donohoe *et al*, 2001; Horbach *et al*, 1996). Serum samples obtained from 20 out of 68 ET patients (29%) were shown to contain APAs, and of those, 10 (50%) had had a thrombotic event. Of the 48 patients without detectable APAs only 12 (6%) had had a thrombotic event (p=0.04) (Harrison *et al*, 2002). APAs may cause persistent activation of the coagulation cascade, inhibit the anticoagulant actions of protein C activation or have a direct effect on platelet activation (Greaves *et al*, 2000). The presence of APAs is unlikely to mark a primary event in the pathogenesis of ET, but may be useful to identify patients at higher risk of thrombosis.

† See additional references p255

Markers of platelet activation A number of groups have investigated markers of platelet activation in an attempt to identify patients at a higher risk of thrombotic events. CD36 (glycoprotein IV) is a platelet receptor that binds collagen and thrombospondin, and as such may be involved in haemostasis and thrombosis. Using a radio-immunoassay a 2-3 fold increase in the number of CD36 molecules at the platelet surface was demonstrated in ET patients compared to normal controls (Thibert *et al*, 1995). Normal controls had a mean 27,500 ± 6160 molecules of CD36/platelet compared to 65,820 ± 29,500 molecules/platelet in 13 ET patients. Patients with a reactive thrombocytosis had similar CD36 levels to control of 26,800 ± 4,500 molecules/platelet, but levels were raised in 10 PV patients (44,960 ± 12,200 molecules/platelet) suggesting that this may be a general marker of cMPD rather than specific for ET.

Vascular endothelial growth factor (VEGF) may be involved in thrombosis formation, and levels of VEGF protein have been shown to be elevated in platelets from ET patients compared to normal controls (Cacciola *et al*, 2003). This elevation was shown to be more severe in patients that had had a thrombotic event than those which had not. Mean VEGF levels were 2.4 ±1.5 pg/10^6platelets for patients with thrombotic events, compared to 1.2 ± 0.8 pg/10^6platelets for patients without thrombotic events.

CD40 ligand is expressed on the surface of activated platelets, and a soluble form of CD40 ligand (sCD40L) can be produced via proteolytic cleavage. An increase in sCD40L levels has been described in plasma from ET patients, but not from normal controls or patients with a reactive thrombocytosis (Viallard *et al*, 2002). There was also a direct correlation between platelet count and sCD40L levels. As platelets shed sCD40L throughout their lifetime, it is possible that this is a reflection of long-lived platelets in patients with ET.

The platelet activation markers P-selectin (CD62p) and thrombospondin (TSP) were elevated in a cohort of 34 ET patients compared to 25 normal controls (Griesshammer *et al*, 1999). The mean percentage of CD62p and TSP positive platelets was 14.7 ± 15.0% and 12.4 ± 9.9% respectively, compared to 3.0 ± 4% and 3.2 ± 3.2% for normal controls. This suggests that increased platelet activation may be a feature of ET. However, in this study no comparison of either CD62p or TSP levels was made between patients who had had a thrombotic event and those which had not had a thrombotic event.

Cytokines The megakaryocytic-derived cytokines platelet derived growth factor (PDGF) -A, -B, and basic fibroblast growth factor (bFGF) have been investigated in a cohort of nine ET patients treated with anagrelide both before and after treatment. All these cytokines are synthesised in the α-granules of platelets and released upon platelet activation. A semi-quantitative RT-PCR method was used to measure levels of mRNA compared to a control gene (Lev *et al*, 2005). The study was able to show that mRNA levels of PDGF A and PDGF B were reduced compared to normal controls prior to treatment, but that levels normalised following treatment, bFGF levels were normal prior to treatment, but also increased after treatment. This group had previously shown a decrease in the levels of PDGF A and PDGF B protein which normalised during treatment, but they also showed an increase in the protein levels of bFGF (Lev *et al*, 2002). These data suggest that the expression of platelet cytokines in these 9 ET patients is aberrant and that this can be partly corrected by treatment.

1.6.6 Correlation of markers

Although a large number of markers for ET, and thrombotic risk in ET, have been described, only a few reports have investigated a number of markers using a single sample cohort. Vannucchi *et al* (2004) investigated PRV-1 over expression, EEC positivity, platelet c-mpl levels and clonality status in a single cohort of 88 ET patients, and correlated the results to the incidence of thrombotic events. They found that while patients with clonal myelopoiesis were at higher risk of thrombotic events, none of the other markers studied showed a significant association with the incidence of thrombosis in the cohort. They were also unable to show a significant clustering of markers in specific patients. A second group has investigated Mpl levels, PRV-1 positivity, EEC formation and the loss of heterozygosity on the short arm of chromosome 9 (9pLOH), the most common chromosomal abnormality described with PV, in a cohort of 15 ET patients (Kralovics *et al*, 2003). Again, they were unable to demonstrate any significant grouping of these potential molecular markers, only one patient was positive for all markers, 3/15 were positive for decreased platelet Mpl levels, PRV-1 over expression and EEC formation, but 2 were negative for all markers studied. These data suggest that ET is a very complex and heterogeneous disorder and that more work is needed to positively identify significant molecular markers which can be used for diagnosis and/or for identifying patients at greater risk of thrombotic events.

1.7 Aims

The aim of the work presented in this thesis was to investigate the pathogenesis of ET in an attempt to further characterise existing molecular markers and identify novel molecular markers which may be of use in the diagnosis of ET or in identifying those patients at higher risk of thrombotic events. Firstly, the existing radiolabelled clonality assay previously used in the laboratory was converted to an assay which used a fluorescently labelled primer. This assay was then used to asses the long-term clonality status of ET patients. Secondly, the 5' UTR of the TPO gene was investigated for the presence of mutations which may effect TPO expression levels, and polymorphisms which may influence the rate of thrombopoiesis were investigated in the TGFβ1 and IL-6 genes. Finally, representational difference analysis (RDA) was used in an attempt to identify novel molecular markers in ET by comparison of the global gene expression levels of a clonal ET patient compared with a normal control at the mRNA level.

Chapter 2

Materials and Methods

2.1 General materials and reagents

Phosphate Buffered Saline (PBS)	Invitrogen
Ficoll-paque™	Amersham Pharmacia
RPMI 1640 medium with L-glutamine	Invitrogen
Foetal Calf Serum (FCS)	Invitrogen
Anti CD3 magnetic beads	Dynal
Dextran T500	Amersham Pharmacia
Penicillin and streptomycin	Invitrogen
NaCl	VWR International
Tris (Hydroxymethyl) methylamine	VWR International
Orthoboric acid	VWR International
EDTA (ethylenediaminetetra acetic acid-disodium salt)	Sigma
DTAB (dodecyl-trimethyl ammonium bromide)	Sigma
TRIzol reagent	Invitrogen
2-ME (β-mercaptoethanol)	VWR International
Ethanol (AnalaR)	VWR International
Isopropanol (AnalaR)	VWR International
Chloroform (AnalaR)	VWR International
Sodium acetate	VWR International
SDS (sodium dodecyl sulphate)	Sigma
Agarose	Sigma/Bioline
Low melting point agarose	Invitrogen
Ethidium Bromide	Invitrogen
Polyacrylamide	National Diagnostics
TEMED (tetramethylethylenediamine)	Sigma
APS (ammonium persulphate)	Sigma
PCR primers	Invitrogen/Oswel
Restriction enzymes / buffers	New England Biolabs / Roche
3MM Chr chromatography paper	Whatman

Hyperfilm	Amersham Pharmacia
TA cloning kit	Invitrogen
Taq polymerase	Promega/Bioline
Taq buffer	Promega/Bioline
dNTPs	Promega/Bioline
Magnesium Chloride	Promega
T4 Polynucleotide kinase	Promega
Reverse Transcriptase	Promega
RNase inhibitor	Promega
DNace quick clean	Bioline
L-Broth	Bio 101 Inc
Agar	Calbiochem
Ampicillin	Invitrogen
X-gal	Sigma
IPTG	Sigma

10x TBE Buffer (pH 8.3). For 1L	-Tris	108.9g
	-Orthoboric acid	55.7g
	-EDTA	7.4g
DTAB (8%). For 250ml	- DTAB	20.0g
	- NaCl	22g
	- EDTA	4.65g
	- Tris	3.05g
10% Dextran. For 250ml	- Dextran 500	25g
	- NaCl	2.25g

2.2 Methods

2.2.1 Isolation of blood cell types

General Method Approximately 32ml of peripheral blood, collected using EDTA as an anticoagulant, was obtained with informed consent and centrifuged at 200g for 15 minutes. The upper layer of platelet rich plasma was removed for isolation of platelets. PBS was added to the remaining blood until the original volume was reached and dextran (10%) added to a final concentration of 1%. Sedimentation of the RBCs occurred after approximately 15 minutes and the top layer, containing the white blood cells, was removed and layered onto Ficoll-paque^{TM} plus. To maximise mononuclear cell (MNC) recovery, the remaining blood was centrifuged at 3,000rpm for 5 minutes and buffy coats collected. These were diluted with PBS to 14ml, and layered onto Ficoll-paque^{TM}. Because of the high red cell content of the buffy coat preparations, no neutrophils were prepared from them. The Ficoll-paque^{TM} preparations were centrifuged at 800g for 20 minutes. The remainder of the method is outlined below in *Platelets, CD3+ cells,* or *Neutrophils.*

Platelets The platelet rich plasma was centrifuged at 2,000g for 15 minutes. The supernatant was discarded and the platelet pellet carefully resuspended in PBS containing 10mM EDTA. RBC contamination of the platelets could be seen at the base of each pellet. Resuspending only the upper portion of each pellet reduced contamination of the platelet sample by RBC. The resuspended pellet was transferred to a new 15ml tube and spun at 2,000g for 15 minutes. This wash procedure was repeated twice. After washing, the platelets were counted, centrifuged at 2,000g for 15 minutes and resuspended in TRIzol at $2x10^9$platelets/ml for RNA extraction. TRIzol preparations were stored at -20°C.

CD3+ cells After centrifugation of the Ficoll-paque^{TM} preparations, the MNC layer at the interface between the upper aqueous layer and the lower Ficoll-paque^{TM} layer was harvested. To remove the Ficoll paque^{TM}, the MNCs were diluted in at least 2 volumes of RPMI 1640 medium with L-Glutamine, supplemented with 2% FCS ($RF_{2\%}$), and centrifuged for 6 minutes at 1,800rpm to remove the Ficoll-paque^{TM}. The pellets were resuspended in $RF_{2\%}$ and pooled, to a final volume of 10ml. Anti-CD3 beads were added to

the MNCs (at a ratio of 1 bead to 1 MNC) followed by incubation for 1 hour at 4°C with end over-end-rotation. After incubation, CD3+ cells were isolated using a magnet and washed 3 times in $RF_{2\%}$. For DNA, 10-50x10^6 cells were re-suspended in 2.4ml $RF_{2\%}$. For RNA, cells were resuspended in TRIzol at 5x10^6/ml. To enable the purity of the sample to be measured, 5x10^4 cells were added to 300µl $RF_{2\%}$ and 2 cytospins prepared.

Neutrophils After centrifugation of the Ficoll-paque™ preparations and removal of the MNC layer, all remaining Ficoll-paque™ was discarded. The resultant pellet was resuspended to a volume of 2.5ml in PBS and 8ml ice-cold water added to lyse the red cells. After 60 seconds, 4ml 3.5% NaCl was added to prevent lysis of the neutrophils. The cells were then spun at 800g for 5 minutes and pellets resuspend in PBS. For DNA 10-50x10^6 cells were re-suspended in 2.4ml PBS. For RNA, cells were resuspended in TRIzol at 5x10^6/ml. To enable the purity of the sample to be measured, 5x10^4 cells were added to 300µl of $RF_{2\%}$ and 2 cytospins prepared.

2.2.2 DNA Extraction

(Adapted from(Gustincich *et al*, 1991). Neutrophils or T-cells (10-50x10^6) were suspended in 2.4ml PBS or $RF_{2\%}$ respectively. DTAB reagent (4.8ml) was added, mixed thoroughly and incubated at 68°C for 5 minutes. After cooling, samples were stored at -20°C for >17 hours. Once thawed, an equal volume of chloroform (7.2ml) was then added to the cells, mixed thoroughly and centrifuged at 2,000g for 20 minutes. The upper aqueous layer was decanted into a new 15ml centrifuge tube and the DNA precipitated with an equal volume of ethanol (100%). The DNA precipitate was then transferred to a 1.5ml tube and washed in 0.5ml 75% ethanol. The DNA was allowed to air dry for approximately 5 minutes before being resuspended in an appropriate volume of water and dissolved thoroughly, by end-over-end rotation, overnight at 4°C. DNA samples were stored at 4°C.

2.2.3 RNA Extraction

Neutrophils or T-cells (5x10^6) were suspended in 1ml of Trizol reagent and stored at -20°C for >17 hours. Once thawed, 200µl chloroform was added and mixed well by shaking for 15 seconds. Samples were incubated at room temperature for 2-3 minutes, and then centrifuged at 15,000g for 15 minutes at 4°C. The upper aqueous phase was

transferred to a clean 1.5ml tube and the RNA precipitated with 0.5ml isopropanol. The samples were then centrifuged at 15,000g for 10 minutes at 4°C, the supernatant discarded and the pellet washed in 300µl 70% ethanol. The samples were centrifuged at 15,000g for 5 minutes at 4°C and the supernatant removed by aspiration. Pellets were allowed to air dry for 5 minutes before being resuspended in an appropriate volume of water. Once dissolved, RNA samples were stored at -70°C.

2.2.4 Agarose gel electrophoresis

Agarose gels (1.0 - 3.5%) were used to check PCR products for size and purity prior to sequencing and to identify size differences after restriction enzyme digestion. The appropriate amount of agarose was added to 35ml of 1x TBE, heated in a microwave oven until dissolved and allowed to cool before the addition of 5µl ethidium bromide (1mg/ml). The gel was poured into a mould and allowed to set. Appropriate volumes of PCR products or restriction enzyme digestions were mixed with loading buffer (30% glycerol, 0.025% Bromophenol blue), loaded into the wells and electrophoresed in 1x TBE (containing 200ng/ml ethidium bromide) under appropriate current and voltage conditions. PCR products were visualised on a UV transilluminator. Polaroid photographic equipment was used to make a permanent record.

2.2.5 Polyacrylamide gel electrophoresis

Denaturing polyacrylamide gels were used for quantitative RT-PCR analysis and non-denaturing gels for RT-PCR-SSCP analysis. Denaturing gels consisted of 6% polyacrylamide (37.5:1 acrylamide to bis acrylamide) in 0.5xTBE containing 8M urea. Non-denaturing gels were prepared in the same way but without urea. To catalyse the acrylamide polymerisation 50µl TEMED and 250µl 10% APS were added to 50ml of gel immediately prior to pouring. Before loading, PCR products were mixed with loading solution (95% formamide, 20mM EDTA, 0.05% Bromophenol blue, 0.05% xylene cyanol), incubated for 5 minutes at 95°C to denature and quenched on ice. The samples were electrophoresed in 0.5xTBE under appropriate current and voltage conditions. After electrophoresis, gels were transferred to 3MM Chr chromatography paper, dried and exposed to autoradiography film for visualisation.

2.2.6 Reverse transcription (RT)

Reverse transcription reactions convert RNA into complementary DNA (cDNA) to enable its use as template in polymerase chain reactions (PCR). RTs were carried out in a 20µl reaction volume, and the final concentrations of each reagent were 1x Bioline reaction buffer (160mM $(NH_4)_2SO_4$, 67mM Tris-HCl, 0.1% Tween-20), 5.25mM $MgCl_2$, 1mM each dNTP, 20 units RNase inhibitor (Promega), 3.75 units reverse transcriptase (Promega), 250ng oligo dT and 1µg RNA. Template RNA and oligo dT in a volume of 12.9µl were incubated for 5 minutes at 65°C followed by 10 minutes at room temperature. This allowed denaturation of RNA secondary structure and binding of the oligo dT primer. The remaining reaction components were then added and the reaction mixture incubated at 42°C for 1 hour followed by 95°C for 5 minutes to denature the enzyme. The cDNA was stable for several weeks when stored at 4°C.

2.2.7 Polymerase chain reaction (PCR)

The PCR allows the amplification of sequence-specific portions of DNA by successive cycles of denaturation, sequence specific primer annealing and DNA polymerisation (primer extension). Two different Taq polymerase enzymes were used during this work, Taq DNA polymerase (Promega) and Biotaq^{TM} DNA polymerase (Bioline). To ensure identical concentrations of each reagent in each reaction tube, a master mix of the reagents was made (minus the template and Taq polymerase) with final concentrations of 1x reaction buffer (Bioline: 160mM $(NH_4)_2SO_4$, 67mM Tris-HCl, 0.1% Tween-20, or Promega: 50mM KCl, 10mM Tris-HCl, 1% Triton X-100) 1.0-2.5 mM $MgCl_2$ optimised for maximal amplification, 200µM each dNTP, 32pmol each of forward (5') and reverse (3') oligonucleotide primer and water to a final volume of 17µl or 18.5µl, and either cDNA (2µl) or DNA (0.5µl) added. A drop of mineral oil was added to each tube as an evaporation barrier. Where a hot-start method was employed, the tubes were placed in the thermocycler block and heated to 95°C for 5 minutes. The temperature was then lowered to 85°C and 1µl of hot start mix, containing 0.5U of Taq DNA polymerase, was added to each tube prior to thermal cycling. Thermal cycling consisted of a denaturation step at 95°C for 30 seconds, a primer-annealing step where temperature was dependent on the composition of the primers for 30 seconds, and a primer extension step at 72°C for 30 seconds. The number of amplification cycles used varied between 22 and 35. A final

extension was carried out at 72°C for 5-15 minutes to ensure complete extension of all PCR fragments.

2.2.8 End-labelling of primers

One primer from a given primer pair for SSCP and semi-quantitative RT-PCR were labelled with a radioactive isotope to allow visualisation on autoradiography film. Ten pmol of primer was incubated with 10 units of T4 polynucleotide kinase in 1x reaction buffer (70mM Tris-HCl, 10mM $MgCl_2$, 50mM DDT) and 3µl γ-[^{32}P]-ATP (0.37 MBq/µl)(Amersham Pharmacia) for 30 minutes at 37°C. This reaction transfers the γ-phosphate from the γ-[^{32}P]-ATP to the polynucleotide primer. After the transfer reaction was complete, samples were incubated at 95°C for 5 minutes to inactivate the enzyme. The labelled primer was then added to the hot start mix for addition to PCRs.

2.2.9 Sequencing of PCR products

PCR products were purified using Wizard Plus PCR™ columns (Promega) or Qiaquick PCR purification kit (Qiagen). Cycle sequencing was performed by addition of 10-100ng purified PCR product and 3.2pmol of an oligonucleotide primer to BigDye™ Terminator Cycle Sequencing Ready Reaction, 310 Genetic Analyser Kit (Applied Biosystems), a single reaction mix that contained the dye terminators, DNA polymerase, $MgCl_2$ and reaction buffer. Twenty five amplification cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes each were carried out on a Perkin Elmer GeneAmp PCR system 9600 thermocycler (Applied Biosystems). The cycle sequencing products were precipitated using 2.0µl Na acetate (3M, pH 4.6) and 50µl ethanol (95%) and incubated at room temperature for 10 minutes. The DNA precipitate was pelleted by centrifugation at 15,000g for 20 minutes. The supernatant was removed and the pellet washed in 75% ethanol. After further centrifugation for 5 minutes at 15,000g, the ethanol was discarded and samples allowed to air dry for 15 minutes prior to resuspention in 15µl Template Suppression Reagent™ (Applied Biosystems). Sequencing results were visualised using the ABI Prism™ 310 Genetic Analyser (Applied Biosystems), a single capillary, fluorescence based, automated genetic analyser. Samples were denatured at 95°C for 5 minutes and quenched on ice prior to electrophoresis on the 310 genetic analyser using performance optimised polymer 6.

2.2.10 Cloning of PCR products

PCR products were cloned using the TA cloning system that utilises the single deoxyadenosine overhang at the 3' end of PCR products. Fresh PCR products were ligated into pCR®2.1-TOPO vector with deoxythymidine overhangs at the insertion site. This was performed by incubating 0.5 - 4μl PCR product with 1μl vector and 1μl salt solution (200mM NaCl; 10mM $MgCl_2$) in a final volume of 6μl at room temperature for 5 minutes. The vector was transformed into TOP10F' competent bacteria by heatshock at 42°C for 30 seconds. LB agar plates (1x LB medium, 15% agar, 100μg/ml ampicillin) were pre-coated with 40μl IPTG (100mM) and 20μl X-Gal (50mg/ml) which was allowed to absorb for 30 minutes at 37°C. Transformed bacteria were selected by resistance to ampicillin and the presence of an inserted PCR sequence was selected by β-galactosidase activity after plating and incubation at 37°C overnight. Transformed clones with inserted sequence (white colonies) were picked and expanded in LB medium containing 100μg/ml ampicillin. PCR was performed directly on the bacteria to select clones for sequencing.

2.2.11 Mutation specific enzyme digests

An appropriate volume of PCR product was digested overnight at the manufacturers specified temperature with 1U/μl of restriction enzyme in the appropriate buffer. Samples were then electrophoresed in 1.5 to 3.5% agarose gels and bands visualised by ethidium bromide staining of the gels.

Where a direct digest was not available to identify sequence changes, a mismatch primer could often be designed to introduce base changes into PCR products and enable restriction enzyme analysis. A mismatch primer is so called because one or more of the nucleotides in the primer sequence are altered from the published sequence. The mismatched bases must not reduce the ability of the primer to anneal to its specific location in the genome or prevent the Taq polymerase from recognising a double stranded primer site, therefore the extreme 3' base pair cannot be changed and a longer primer than is usual is often designed. As the alteration is in the primer sequence, all subsequently produced PCR products will faithfully copy the mismatch leading to the introduction of the alteration into all exponentially amplified products. Enzyme digestions are then carried out as described above. As the enzyme cutting site is partially within the primer sequence, the use

of a long primer (>30bp) also facilitates separation of the bands representing cut and uncut PCR products when visualised using agarose gels.

Chapter 3

Optimization of PCR based clonality assays using a fluorescently labelled primer

3.1 Introduction

3.1.1 Clonality in cell lineages

Expansion of a specific cell lineage can arise in either a clonal or polyclonal fashion. Alterations acquired in the DNA of a single cell may lead to a growth and/or survival advantage for that cell and its progeny, giving rise to a clonal expansion of the cell lineage. However, expansion may also occur by over-stimulation of cell proliferation by exogenous factors, such as cytokines or growth hormones, leading to a non-clonal, or polyclonal, expansion of the cell lineage. Understanding the clonal basis of the expansion is fundamental to its study.

A number of strategies are available to study clonality. If a chromosomal abnormality is associated with a disease, karyotype analysis can be used. Nearly all classical CML patients have a t(9;22) translocation, termed the Philadelphia chromosome (Ph^1), in 90-100% of their BM cells (Guilhot, 1999). As haematologically normal controls display no Ph^1 it can be used as a marker for the clonal expansion of the myeloid lineage in CML. The study of oncogenic mutations can also be used to determine the clonality of neoplasia. Mutations of the RAS superfamily of GDP/GTP-binding proteins have been identified in 15-25% of AML cases where they can be used as a marker for diagnosis and clonality in those patients (Farr *et al*, 1988; Stirewalt *et al*, 2003). Immunoglobulin gene rearrangements can be used to identify clonality in some diseases, particularly in lymphoid malignancies. A normal population of B or T-cells will contain a large number of gene rearrangements giving a diversity of immunoglobulins or T-cell receptors. In lymphoid neoplasia this diversity is often reduced to a single or predominant immunoglobulin or T-cell receptor (TCR) indicating a clonal expansion (Gilliland *et al*, 1991a). However, infection can also lead to a clonal expansion of a single or predominant TCR or immunoglobulin, so results must be interpreted with care.

In diseases where a direct marker of clonality is not known, clonality can be measured in female patients by indirect means using X-chromosome inactivation patterns.

3.1.2 Lyonization and XCIPs

All mammalian female somatic cells have one active and one inactive X-chromosome which prevents a lethal double dose of genes from the X-chromosome.

Inactivation occurs during embryogenesis where either the maternal or paternal X-chromosome is inactivated at random. This process is known as Lyonization (Lyon, 1961). Once inactivation has occurred it is fixed and the same inactive X-chromosome is inherited in all progeny cells even if the cell becomes neoplastic (Gartler & Riggs, 1983) (Figure 3.1). Therefore, if the expansion of a cell lineage is derived from increased proliferation/survival of a single cell, all cells of that lineage would have the same active X-chromosome leading to an imbalanced XCIP. To use Lyonization as a marker for clonality the active and inactive X-chromosome must be able to be identified, as well as the maternal and paternal X-chromosomes. Protein variants and polymorphisms of genes located on the X-chromosome enable this distinction to be made. The following assays have proved to be useful tools for investigating the clonal derivation of a cell population.

3.1.3 Studying clonality with protein isozymes

The earliest use of XCIPs to determine clonality used the housekeeping gene glucose-6-phosphate dehydrogenase (G6PD) which has been used to study the cellular origin and development of a number of different tumours (Beutler *et al*, 1962; Fialkow, 1976; Raskind & Fialkow, 1987). A number of G6PD protein variants occur in different ethnic populations, the most common of which is type B G6PD. Most females are homozygous for type B, and this lack of heterogeneity limits the usefulness of the technology. However, 30-40% of males of African origin have a variant enzyme, type A (Fialkow, 1972). The A and B forms differ by one amino acid substitution and are readily distinguishable using electrophoresis (Boyer *et al*, 1962). Thus heterozygous females can be studied, a clonal population being indicated by a single band in the neoplastic cell lineage, and a polyclonal population indicated by two bands (Beutler *et al*, 1962). As the technique used protein as the test material, the clonality of non-nucleated cells, for example RBCs or platelets, could be deduced.

3.1.4 Studying clonality with DNA assays

3.1.4.1 Southern blot techniques

The use of G6PD isozymes to measure XCIPs is restricted as few patients are heterozygous. Therefore other assays were developed to allow study of a larger number of individuals. No other protein isozymes have been identified to date that can be used for clonality assays. However, the discovery that inactivation of the X-chromosome is

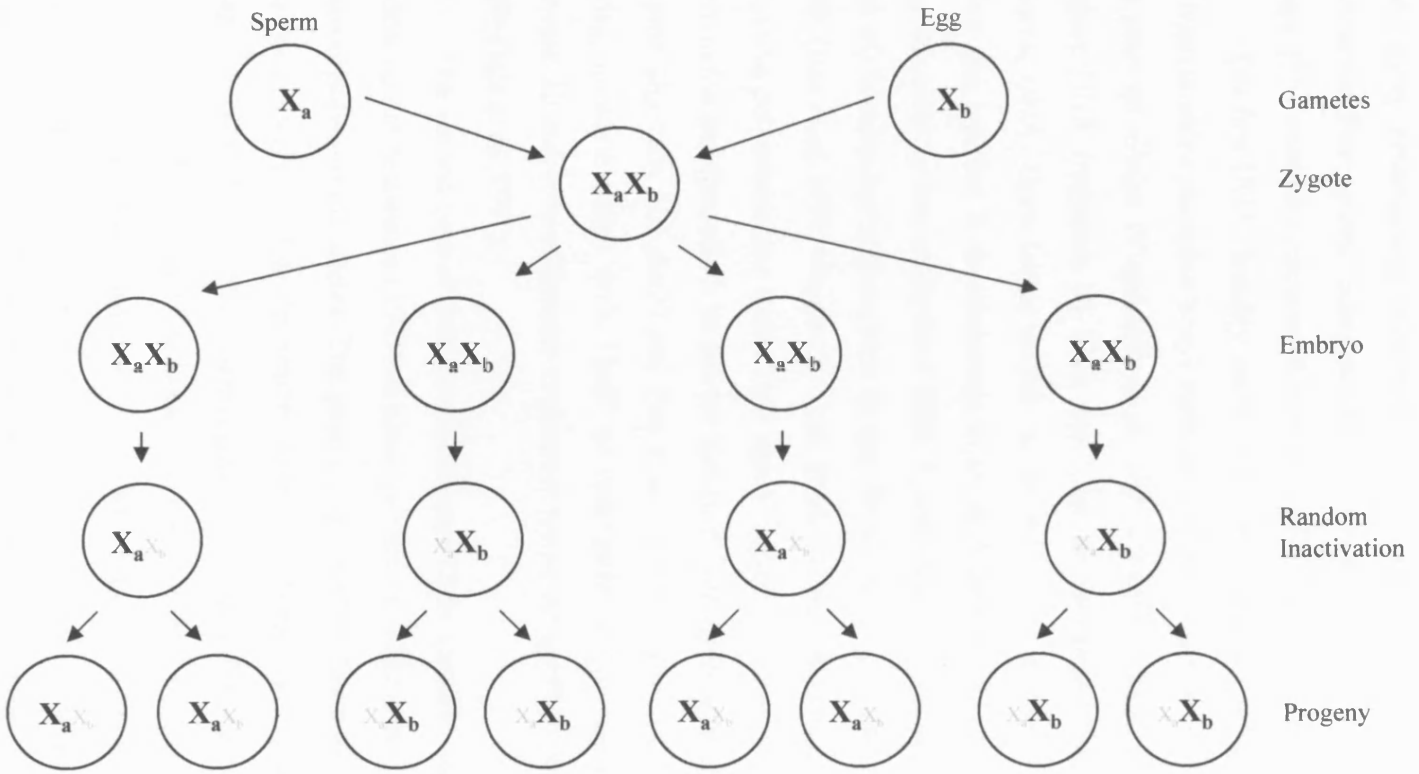

Figure 3.1
Schematic of X-chromosome inactivation
Active X-chromosomes shown in large, bold, black type, inactivated chromosomes in small grey type. X_a represents paternal chromosome, X_b represents maternal chromosome.

accompanied by DNA methylation of specific cytosine residues within certain genes enabled DNA techniques to be developed (Razin & Riggs, 1980). By using DNA polymorphisms, to distinguish between the two X-chromosomes, and differential methylation, investigators were able to examine clonality in a much larger number of individuals. Two general techniques have been established for Southern blot based clonality assays: (1) – restriction enzyme digestion and (2) - short tandem repeat (STR) based assays.

The first DNA clonality assay used a polymorphic restriction enzyme cutting site in the hypoxanthine phosphoribosyl transferase (HPRT) gene to distinguish between maternal and paternal alleles (Vogelstein *et al*, 1985). Females polymorphic at the HPRT locus produce DNA fragments of 18kb and 12kb by Southern blot after *Bam*HI digestion of genomic DNA. These bands contain six methylation-sensitive *Hha*I sites which allow the active and inactive X-chromosomes to be distinguished. Methylation-sensitive restriction enzymes only cut non-methylated DNA so only active alleles are digested. Between 12 and 27% of females are polymorphic at the HPRT locus (Anger *et al*, 1990; Browett *et al*, 1988; Gale *et al*, 1991; Vogelstein *et al*, 1985). At the phosphoglycerate kinase (PGK) gene locus two polymorphisms in the CpG rich 5' region are close to sites that show differential methylation and thus allow its use for clonality studies (Vogelstein *et al*, 1987). Restriction enzyme digestion with *Bst*XI and *Pst*I is used to distinguish between the two polymorphic alleles, in combination with *Hpa*II to distinguish active and inactive X-chromosomes. Between 30 and 40% of females are heterozygous for the PGK polymorphism (Anger *et al*, 1990; Gale *et al*, 1991).

The second type of Southern blot based DNA clonality assay uses variable number tandem repeat sequences (VNTRs) close to sites of differential methylation to distinguish between polymorphic alleles. The gene locus DXS255, identified by the probe M27β, has also been widely used to determine clonality (Abrahamson *et al*, 1990; Boyd & Fraser, 1990; Fey *et al*, 1992). The heterozygosity rate for M27β can be as high as 93%, much higher than for HPRT, and has been used successfully by a number of groups (Fey *et al*, 1994; Gale *et al*, 1992). However, use of M27β has been hampered because the extent of methylation of inactive alleles is variable at this locus, leading to XCIPs that can be very difficult to interpret with the methylation-sensitive restriction enzyme *Hpa*II (Hodges *et al*, 1991). This problem can be reduced by using *Hha*I for digestions (Gale & Linch, 1994b).

3.1.4.2 PCR Techniques

Southern blot analysis is time consuming and labour intensive and typically 5-20μg of DNA is required to generate the high levels of signal required. To allow clonality to be studied more easily, and in individual haematological cell lineages where the quantity of DNA available is often small, PCR technology has been used to amplify the DNA. Most studies to date have used polymorphic loci in either the human androgen receptor (Allen *et al*, 1992; Gale *et al*, 1996) or PGK gene (Gilliland *et al*, 1991b; van Kamp *et al*, 1991). PCR based assays rely on the proximity of the methylation-sensitive restriction sites to polymorphisms, allowing relatively short PCR products to be used.

The human androgen receptor assay (HUMARA) utilizes an STR in the coding region of the human androgen receptor gene which is close to a differentially methylated *Hpa*II restriction site. The STR is 3bp in length and is highly polymorphic, with one study describing 20 different alleles corresponding to 11-31 CAG repeat units, and a heterozygosity level of 90% (Allen *et al*, 1992). The STR generates a size difference enabling identification of the polymorphic alleles. Samples are digested with *Hpa*II prior to amplification as PCR does not preserve methylation. Unmethylated DNA is cut between the two primer annealing sites, preventing PCR amplification (Figure 3.2). However, methylated DNA remains uncut and the inactive X-chromosome is amplified. As the difference between alleles can be as little as three base pairs, and the results obtained require quantification, either isotope labelled PCR amplification and electrophoresis through acrylamide gels, or fluorescent labelled PCR amplification and the use of a genetic analyser, is required.

The PGK assay is similar to the HUMARA but identification of the polymorphic alleles occurs via the use of a single nucleotide polymorphism (SNP). Samples are digested with *Hpa*II prior to PCR amplification to cut the DNA between the two primer sites thus preventing amplification of active alleles. After amplification, PCR products are digested with *Bst*XI to distinguish between the polymorphic alleles. Isotope-labelling and densitometry, or fluorescently labelled PCR and the use of a genetic analyser, allow quantification (Gilliland *et al*, 1991b).

3.1.5 Studying clonality with RNA assays

DNA methods, especially the HUMARA, have been widely used to measure XCIPs because of high heterozygosity rates in the population. However, only nucleated cells can

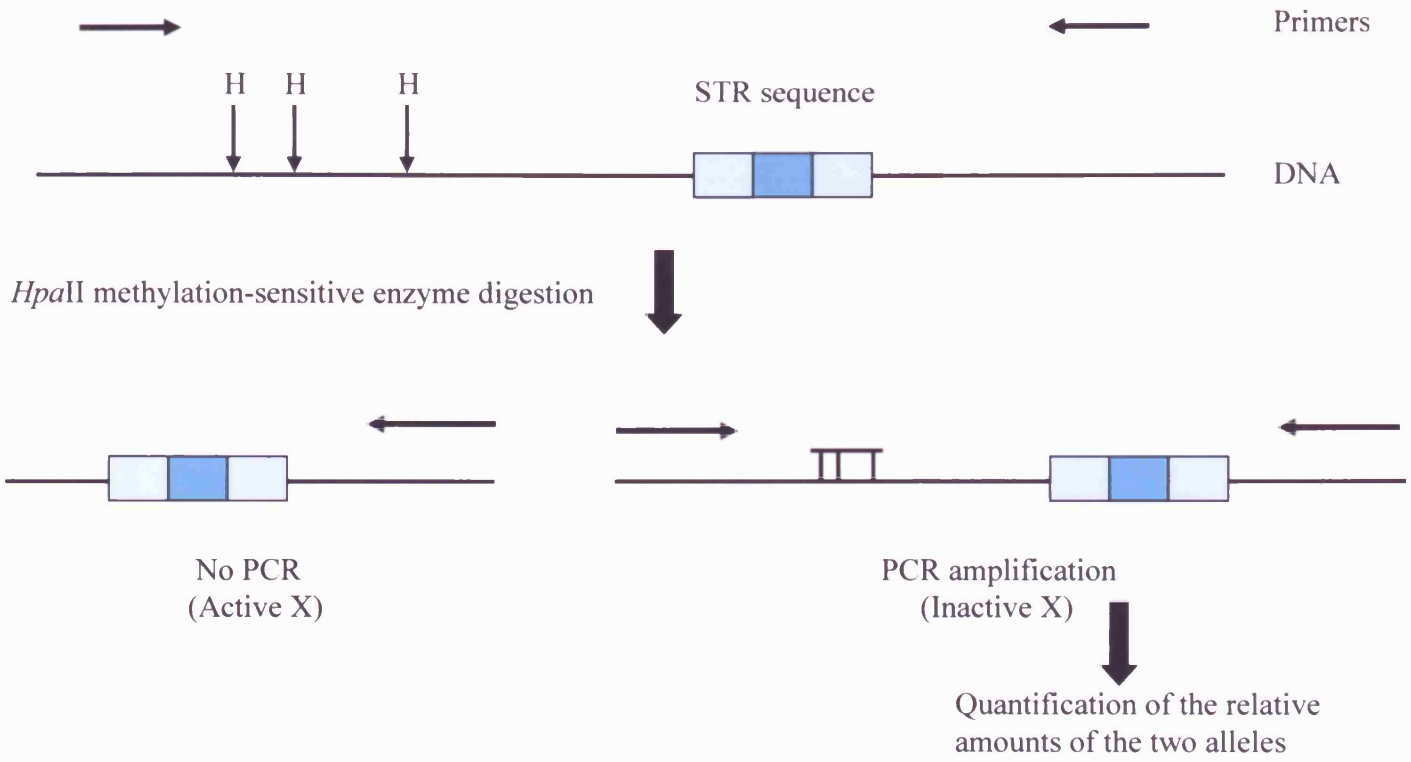

Figure 3.2
The HUMARA
Differential amplification of active and inactive X-chromosomes using the HUMARA
H=*Hpa*II restriction enzyme cutting sites

be studied with DNA techniques and the polymorphism at a DNA locus must be near to a differentially methylated site which can be recognized by a methylation-sensitive restriction enzyme. Also methylation patterns at some loci are complex due to sites with variable methylation (Gale & Linch, 1994b) and both hyper- and hypomethylation have been documented in tumour samples and cMPDs (Herman & Baylin, 2003; Ihalainen *et al*, 1994; Jones *et al*, 2004).

To avoid these problems RNA based clonality assays have been developed that directly measure transcript levels of polymorphic genes located on the active X-chromosome. They circumvent the problems associated with techniques using methylation and allow measurement of clonality in non-nucleated cells such as reticulocytes and platelets. Any SNP present in mRNA derived from the X-chromosome can be used for the RNA assays so long as it is expressed in the cells being investigated. As with DNA, RNA can be extracted from small numbers of cells, which allows measurement of clonality from specific cell lineages where few cells may be available. To prevent contaminating DNA in the RNA preparations interfering with quantification, PCR primers are designed which cover exon/intron boundaries. This ensures that contaminating DNA either does not amplify, or produces a PCR product larger than that from RNA.

A number of polymorphic genes on the X-chromosome can be used to study clonality at the RNA level. The first of these uses the polymorphic G6PD housekeeping gene. A ligase detection reaction was originally used to study the G6PD polymorphism (a C to T substitution at nucleotide 438) (Prchal & Guan, 1993). However, a simpler mismatch primer (Chapter 2 section 2.2.8) and labelled PCR, followed by *Mlu*I restriction enzyme digestion can also be used (Harrison *et al*, 1999a).

Two other polymorphic loci are commonly used: iduronate-2-sulphatase (IDS) (Hopwood *et al*, 1993; Harrison *et al*, 1998a), and the palmitoylated membrane protein p55 (Luhovy *et al*, 1995). The IDS assay exploits a C to T substitution in codon 146 of the IDS gene that, by introducing a mismatch with the 3' primer (Figure 3.3), can be cut using *Bcl*I (Harrison *et al*, 1998a). The p55 polymorphism is used in a similar way, by the introduction of a RFLP by the use of a mismatch primer allowing digestion by *Bst*U1 to identify the two alleles.

Two further polymorphic loci in the Bruton tyrosine kinase (BTK) and four and a half LIM domain 1 (FHL-1) genes have recently been described (Liu *et al*, 2003). Up to

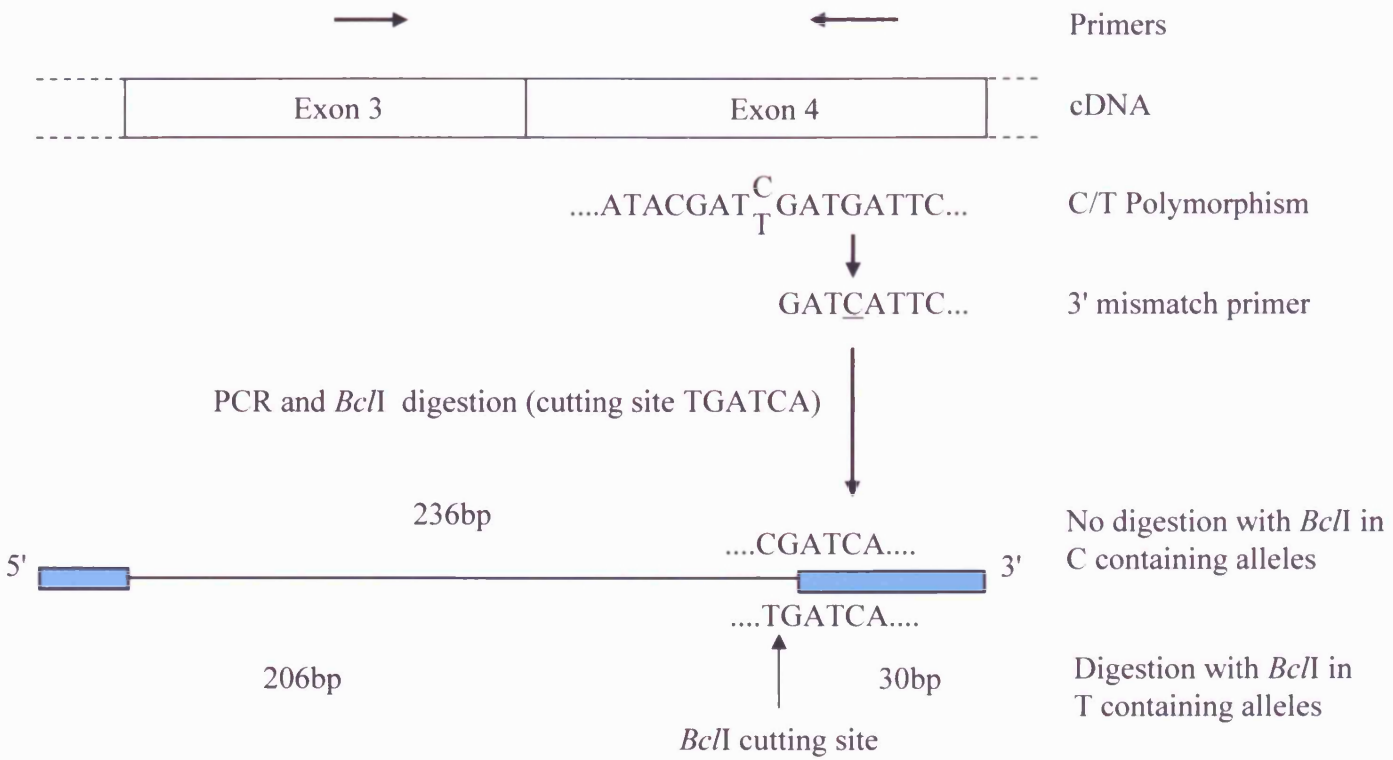

Figure 3.3
The IDS assay
Identification of the two alleles in the IDS assay using a mismatch primer and restriction enzyme digestion

95% of females are informative for at least one of these five loci, enabling clonality at the expression level to be studied in most female patients (Liu *et al*, 2003).

RNA clonality assays are useful tools, but they do have their own specific problems. DNA heteroduplexes can form during the last cycle of PCR amplification as the primers are incorporated into the PCR products and the amount of product increases. This heteroduplex DNA will not have the recognition sequence required for restriction enzyme binding and digestion and so will not be cut by restriction enzymes. This leads to the artificial altering of clonality results towards a balanced XCIP. DNA assays control for this by comparison of two aliquots from each sample, one with and one without *Hpa*II digestion. However, this is not possible for RNA assays as there is only one aliquot per sample. A further concern is the effect of contamination with cells from a different lineage as transcript levels in T-cells, neutrophils and platelets can differ for any given gene. At the IDS loci, transcript levels from one T-cell were equivalent to those from six neutrophils or 800 platelets (Harrison *et al*, 1998a). As clonality may differ between lineages, contaminating cells can affect clonality results.

3.1.6 Measuring clonality

Interpretation of clonality results is required before a sample can be classified as clonal or polyclonal. Cells may be present in a sample which are not derived from the expanding clonal population. This may be because the clone does not have sufficient growth advantage to overwhelm the entire lineage. Therefore, while the tumour may derive from a clonal expansion of a progenitor cell, the clonality assay may still show the presence of both alleles. Both polymorphic alleles were present in tumour samples from 41 patients, but their relative expression had become imbalanced therefore an arbitrary definition of a clonal pattern was defined (Vogelstein *et al*, 1987). An 80% or greater decrease in the intensity of one allele with a decrease in intensity of less than 40% in the other allele following digestion with methylation-sensitive restriction enzymes was suggested as the definition of a clonal XCIP (Vogelstein *et al*, 1987). This equates to less than 20% expression of one allele compared to more than 60% of the other allele or a 1:3 expression ratio. Using this definition, 36 of 41 patients with a neoplastic disorder had a clonal pattern, with the other 5 being uninterpretable by the HPRT assay used in these studies. Normal tissue from 40/41 of the patients had a polyclonal pattern of XCIP using this criteria, the other patient was constitutively skewed (discussed below) (Vogelstein *et al*, 1987). Many

early studies using XCIPs gave results as a ratio of one allele to the other as described above. However, it is now more common to express the ratio as a percentage, with <25% expression of one allele indicating an imbalanced XCIP.

A second consideration is constitutive skewing which is thought to occur because the stem cell pool is small at the time of X-chromosome inactivation. Estimates of the progenitor cell pool size for human haemopoietic cells have ranged from 7 to 16 (Fialkow, 1973; Gale *et al*, 1991; Puck *et al*, 1992; Gale & Linch, 1994a). Therefore, due to the random nature of Lyonization, 20-40% of haematologically normal samples would be expected to have an imbalanced XCIP. To control for constitutive skewing of myeloid lineage cells the XCIP of T-cells is also measured. T-cells are derived from the same embryonic stem cell as myeloid cells and are more appropriate than other tissues such as skin or cultured fibroblasts as a number of studies have indicated that XCIPs are tissue specific (Brown *et al*, 1990; Fey *et al*, 1992; Gale *et al*, 1994).

With the use of T-cell control tissue it has become clear that a caveat must be placed upon the classification of clonal samples. In one study of 24 haematologically normal controls, the results for T-cells and neutrophils differed by 0-13% and 10 out of the 24 samples showed an imbalanced XCIP in neutrophils (Gale *et al*, 1994). Therefore a difference of 20% between T-cell and neutrophil results was arbitrarily chosen as a further requirement for a sample to be classified as clonal.

Several studies have suggested that XCIPs can change with age. In a study of 105 healthy females, significantly more individuals had an imbalanced XCIP in the elderly group (75-96 years) compared to the young group (20-58 years) (Fey *et al*, 1994). Another study showed imbalanced XCIPs in haematologically normal samples in 14/162 (8.6%) neonates, 11/67 (16.4%) subject aged 28-32 years and 22/66 (37.9%) females aged 60 years or over (Busque *et al*, 1996). However, these studies are difficult to interpret as they used DNA from whole blood for the assays. In a cohort of 80 normal individuals aged 75 years and over, 56% had an imbalanced XCIP in the myeloid compartment compared to only 22% of 94 subjects aged 17-50 years (Gale *et al*, 1997). The difference between neutrophil and T-cell results in the elderly group was greater than the 20% variability previously outlined. It was also demonstrated that the imbalanced XCIP associated with age was more pronounced in the myeloid lineage than the lymphoid lineage. Therefore, an upper age limit for a clonal result from clonality assays of sixty five years of age was arbitrarily chosen to exclude patients with age related skewing of the myeloid lineage.

The XCIP based clonality assays are therefore limited in their use. For patients to be entered into a clonality study they must be young females (<65 years), polymorphic for one or more assay, and without a constitutively imbalanced XCIP. Individuals who meet this criteria are defined as clonal when expression of one allele in the myeloid lineage is less than 25% and the difference between T-cell and neutrophil results is greater than 20%.

A technical consideration is also required. To control for incomplete digestion of restriction enzymes in the DNA assays two aliquots are prepared for each sample, one with and one without the methylation-sensitive restriction enzyme. Comparison of the two aliquots allows correction for incomplete digestion.

3.1.7 Drawbacks of a radio labelled assay

Many of the studies described above used a radioactive isotope to enable quantification of clonality results. However, radioactivity is a mutagen, and is therefore highly toxic. It is invisible and requires the use of a Geiger counter for detection. Its use and disposal are highly regulated due to the hazard it poses to the user and the environment, and it is expensive to manufacture. The ^{32}P used in these assays has a short shelf life due to the 14.5 day half life of the isotope and is a high energy β-particle emitter. All handling must therefore be carried out behind 1cm or more of Perspex shielding to protect the user. Visualization may require a number of exposures to autoradiography film to enable reliable densitometry, extending the time taken from sample acquisition to accurate result.

The PCR based assays enable the use of fluorescent labels for quantification rather than radioisotopes. Fluorescent dyes are less hazardous to the user and the environment, and because a shield is not required, their use is less laborious than radioactive isotopes. They are less expensive to purchase, store and dispose of than radioisotopes, and the electrophoresis step can be automated by the use of an automated genetic analyzer eliminating the need for multiple exposures of autoradiography film and densitometry.

3.1.8 Aim

The aim of the work detailed in this chapter was to develop a quantitative, fluorescently labelled clonality assay for DNA and RNA samples, and to ensure results were reliable and reproducible.

3.2 Methods and Results

3.2.1 Calculating clonality

All clonality results obtained during this work are expressed as the percentage expression of the smaller allele. The first stutter peak was included in calculations for HUMARA when there was more than one repeat difference between alleles (see section 3.2.2.3). Results used for comparison with radioactive results were obtained in duplicate and the results expressed as the mean of the two values.

3.2.2 HUMARA

3.2.2.1 Screen

Patients were screened to determine whether they were informative for HUMARA. Previously, this had been achieved by radiolabelled PCR and electrophoresis through acrylamide gels. However, after the initial work detailed in this chapter had been carried out, the fluorescent system was used. Approximately 200ng of DNA was digested with 5U *Rsa*I (Roche) and 1x buffer L in a final volume of 5µl and incubated for 17 hours at 37°C. PCR was then carried out as described in chapter 2 (2.2.7), using the fluorescently labelled primer HUM/F (5'FAM) and the unlabelled primer HUM/R (Table 3.1), at an annealing temperature of 68°C with Promega reagents, and 2µl PCR product was loaded onto the ABI310 with an injection time of 10 seconds. Informative patients have two peaks on electrophoretograms.

3.2.2.2 Radioactive Assay

All radioactive clonality data presented had been previously obtained in the laboratory (Gale *et al*, 1996).

Assay	Forward primer (5'→3')	Reverse primer (5'→3')	Fragment Size (bp)	Annealing Temp (°C)
HUMARA	TCCAGAATCTGTTCCAGAGCGTGC	GCTGTGAAGGTTGCTGTTCCTCAT	Variable	68
IDS (D)	GCCCCAAAGAAGGGAGGGTCC	TGGAAAAGACCAGCTATACGGAGAATGATC†	160	64
IDS (R)	TTTGCGCAGCAAGCAGTGTGCG	TGGAAAAGACCAGCTATACGGAGAATGATC†	236	64

Table 3.1
PCR primers and fragment sizes for HUMARA and IDS clonality assays and IDS heterozygosity screen.
Both DNA (D = heterozygosity screen) and RNA (R = clonality assay) sizes are shown for the IDS assays as well as the appropriate annealing temperature for each PCR reaction.
† Mismatch primers with mismatched bases underlined

3.2.2.3 Fluorescent assay Optimization

Basic Conditions

To ensure that the fluorescent technique was accurate and reproducible, it was important to establish the basic conditions required for the new technology. The assay was set up with the same initial parameters as the radioactive assay. For each sample two aliquots were prepared for enzyme digestion; one aliquot contained approximately 200ng of DNA with 5U *Rsa*I (Roche) and 1x buffer L, the other aliquot contained approximately 400ng of DNA and 5U of the methylation sensitive restriction enzyme *Hpa*II (Roche) in addition to 5U *Rsa*I and 1x buffer L. Both aliquots were in a final volume of 5µl and were incubated at 37°C for 17h. *Rsa*I was used in both aliquots to fragment the DNA and thus increase the efficiency of the PCR. Twice the amount of DNA was used in the *Hpa*II containing aliquot because *Hpa*II digests non-methylated DNA between the primer annealing sites, and thus no PCR product is produced from the active X-chromosome (50% of the sample). PCR was then carried out as described in chapter 2 using the fluorescently labelled primer HUM/F (5'FAM) and the unlabelled primer HUM/R at an annealing temperature of 68°C with Promega reagents (Table 3.1). The first variable condition investigated was the number of PCR cycles.

Number of amplification cycles required

PCR cycles should be kept as low as possible for the HUMARA. Smaller fragments are preferentially amplified in a PCR reaction, which could skew clonality results in favour of the smaller allele, giving false results; this is of particular importance when the size difference between alleles is large (Gale *et al*, 1996). Also, higher cycle numbers increase the intensity of stutter peaks (Kunkel & Bebenek, 2000). Stutter peaks are caused when the Taq DNA polymerase makes copying errors as it scans the STR and single units of the repeat are lost. This is a rare event, and at low cycle numbers only a small number of extra bands can be seen, 3bp smaller than the main band (Figure 3.4). However, stutter can still cause problems when the two different alleles are only one repeat unit different in size, because in this situation the major stutter peak migrates at the same speed as the smaller allele and interferes with the measurement of the allelic ratio.

To test how many PCR cycles were necessary to give peaks within the limits of detection of the instrument, 3 DNA samples with differing numbers of repeat units between

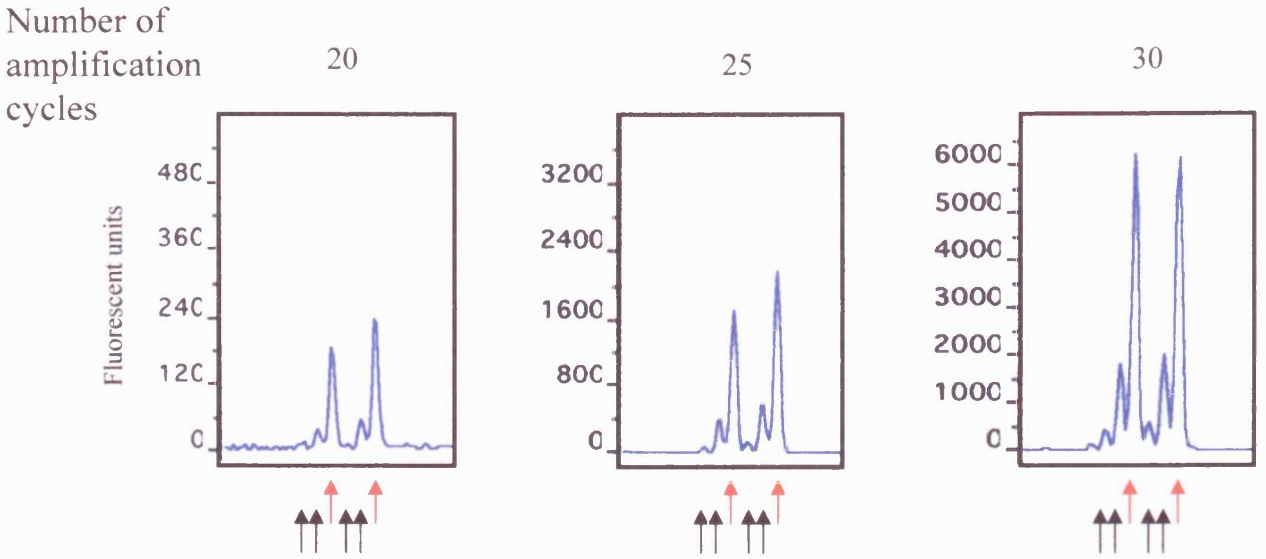

Figure 3.4
HUMARA : The effect on peak height of increasing the number of amplification cycles.
Black arrows indicate stutter peaks, red arrows indicate main sample peaks

alleles (2, 3 and 5) were digested with *Rsa*I only and three separate PCR reactions were carried out using the fluorescently labelled forward primer HUM/F (5'FAM) and HUM/R for 20, 25 or 30 PCR cycles. Samples were loaded onto the instrument in a mix containing 12µl de-ionized formamide, 0.5µl GS500 TAMRA size standard and 1µl of PCR product. Samples were then incubated at 95°C for 5 minutes to denature and quenched on ice. An injection time of 5 seconds was used and a run time of 22 minutes. The results for each sample were similar and displayed the same trend of increasing peak height with increasing cycle number. A representative sample (shown in Figure 3.4) had two repeat units between alleles and both alleles gave approximately equal peak heights. At 20 cycles the highest peak measured approximately 240 fluorescent units (FU), at 25 cycles over 2000FU and at 30 cycles over 6000FU. The instrument's detectors become saturated at 7000FU, so 30 amplification cycles was thought to be too close to the detection limit of the instrument for good reliability. The peak height of approximately 240FU seen with 20 cycles is towards the lower limit of detection and the base line was not flat. The preferred option was 25 amplification cycles as this gave a peak height of 2000FU, with is towards the middle of the detection range of the instrument and gave a flat base line. However, to minimize the number of cycles, and as the previously used radioactive methodology used 20 PCR cycles, other conditions were investigated to try to increase peak height without increasing the number of amplification cycles beyond 20.

Sample volume and injection time required

The volume of PCR product used and the length of time the DNA is electrophoresed onto the capillary (injection time) were investigated for their effect on peak height. One DNA sample was used to test the effect on peak height of loading different volumes of PCR product onto the instrument. Aliquots of 1, 2 or 5µl were taken from a PCR amplified for 20 cycles. Each aliquot was added to 12µl de-ionized formamide and 0.5µl GS500 TAMRA size standard and run on the ABI310 using an injection time of 5 seconds. While a small increase in peak height was seen from 1µl (approximately 250FU) to 2µl (approximately 300FU), this difference was minor and still gave peak heights close to the instrument's detection limit (Figure 3.5). A DNA volume of 5µl gave similar results to 2µl.

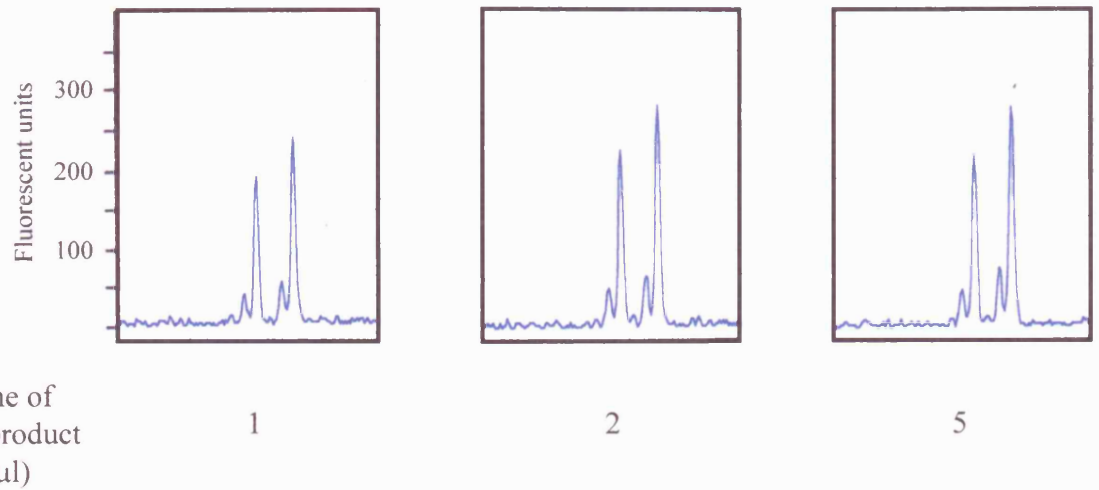

Figure 3.5
HUMARA : The effect on peak height caused by increasing the volume of PCR product loaded

More significant changes in peak height were obtained by altering the injection time. Each DNA sample is loaded onto the polymer by electrophoresis with the capillary end in the sample tube. The capillary end is then transferred to a tube containing TBE buffer for sample separation. The time allowed for this electrophoresis is termed the injection time, and can be altered. Four aliquots containing 1µl PCR product from one DNA sample were injected for 5, 10, 15 or 20 seconds. A 2-fold increase in peak height was seen by increasing the injection time from 5 to 10 seconds (Figure 3.6A) and a smaller, but still significant, increase from 10 to 15 seconds. However, no significant difference in peak height could be seen between 15 and 20 seconds injection times. A graph of peak heights (Figure 3.6B) indicated a plateau effect occurring at an injection time of greater than 15 seconds, suggesting a factor had become rate limiting. This may be because most of the DNA sample had been electrophoresed out of the loading solution, so increasing the injection time further would not increase the amount of DNA loaded onto the capillary.

Increasing both the DNA amount and the injection time may overcome this problem. Three aliquots containing 1µl PCR product from one DNA sample were injected for 5, 10 or 15 seconds and a fourth aliquot, containing 2µl PCR product was injected for 15 seconds. With a 15 second injection time an increase in peak height was seen with 2µl PCR product compared to 1µl, suggesting that the entire sample had indeed been electrophoresed onto the capillary using only 1µl of PCR product and a high injection time (Figure 3.7). However, the height of the peaks was still too near the lower limit of detection to ensure results would be accurate and reproducible. To ensure sufficient PCR product was obtained, PCR amplification were increased from 20 to 22 cycles, and 2µl of PCR product was used with an injection time of 10 seconds.

3.2.2.4 Test Samples

After establishing initial running conditions for the HUMARA it was important to ensure that, not only did these conditions give reproducible results, but also that they were comparable with previously obtained radioactive results. The HUMARA was carried out on a set of 25 samples which included 7 neutrophil samples from AML patients and 18 DNA samples from normal controls. All 25 samples had previously been assayed by radioactive HUMARA and were chosen because their clonality results covered the entire range of results possible, from 0% to 100% lower allele. Two aliquots of each DNA sample were digested with either *Rsa*I alone or both *Rsa*I and *Hpa*II in buffer L (Roche) in a final

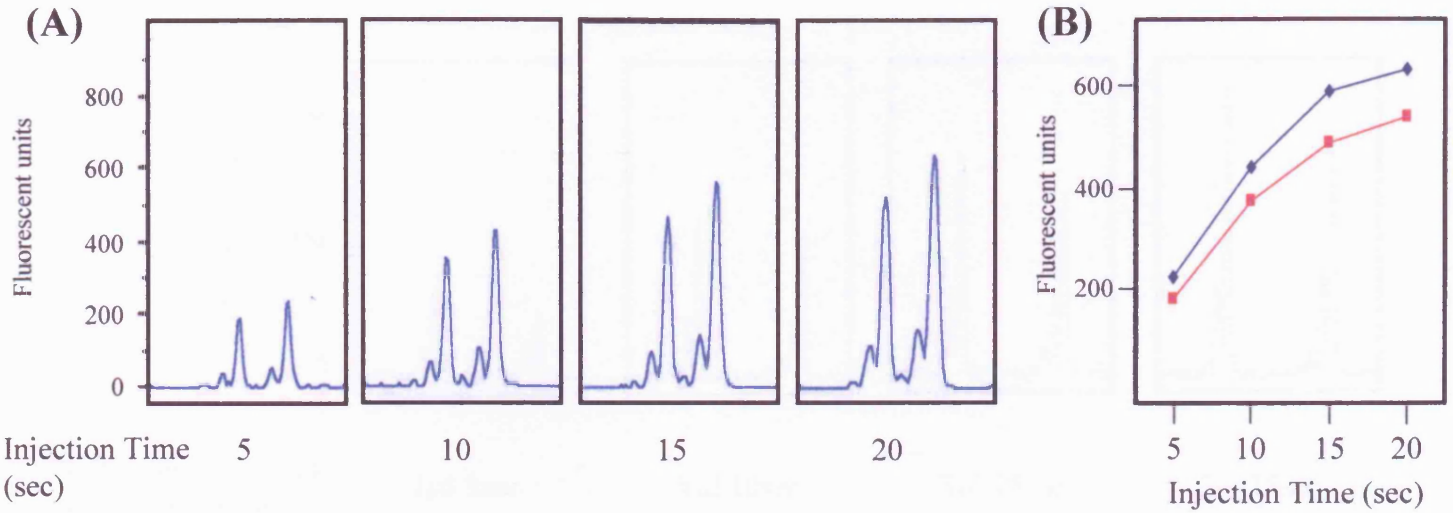

Figure 3.6

HUMARA : The increase in peak height associated with an increase in injection time

(A) - Electrophoretograms of the sample at each injection time

(B) - Graph of the sample peak heights at each injection time. The blue and red traces represent the upper and lower peaks respectively

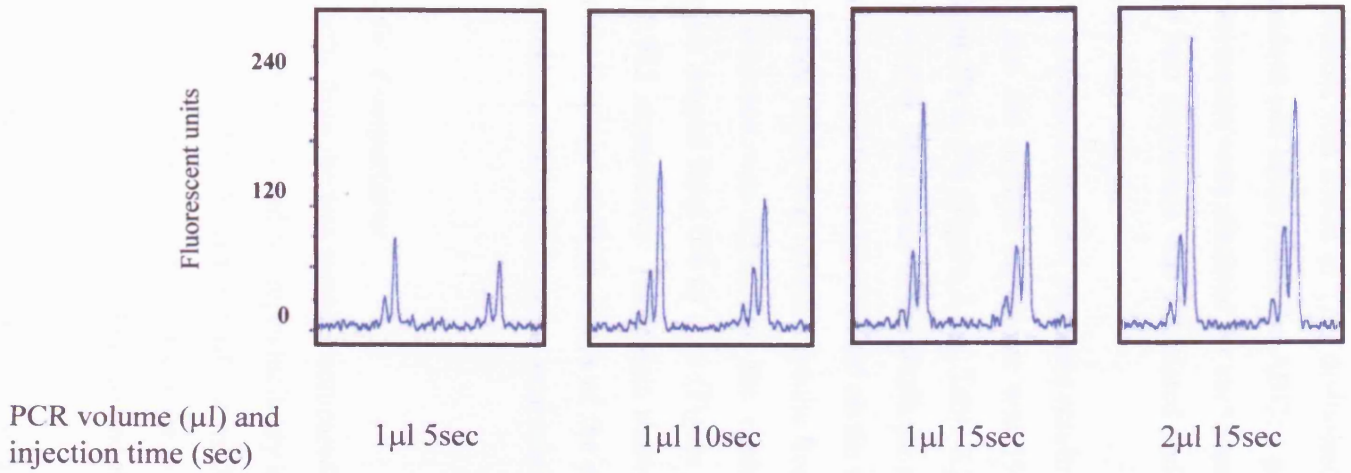

Figure 3.7
HUMARA : The effect on peak height of increasing both DNA loaded and injection time

volume of 5µl for 17 hours at 37°C. PCRs were then set up as outlined in chapter 2 (2.2.7) using primers HUM/F and HUM/R and the 5µl digest reaction as DNA template. Reactions were incubated without Taq 95°C for 5 minutes, followed by addition of 5U Taq (Promega) and 22 amplification cycles at an annealing temperature of 68°C. After amplification a 2µl aliquot of PCR product was added to 12µl de-ionised formamide containing 0.5µl GS500 TAMRA size standard and loaded onto the ABI310 genetic analyser with an injection time of 10sec. Duplicate results were obtained for each sample and compared for reproducibility. The mean of the two duplicates was calculated and compared with previously obtained radioactive data for each sample.

The mode difference between duplicate results of the 25 samples was 0%, the mean difference 1.3%, and the median difference was 1%. The difference between duplicate results ranged from 0% to 4% (Figure 3.8A, Table 3.2). Correlation coefficients were high at r=0.998 and r^2=0.996. The mean of the duplicate results obtained using the fluorescent HUMARA were compared to results obtained on the same set of samples using radioactive HUMARA. The mode difference between results from each HUMARA methodology was 0%, the mean difference was 3.5%, and the median difference was 3%. Differences between each result ranged from 0% to 10% (Figure 3.8B, Table 3.2). Values for r and r^2 were 0.992 and 0.983 respectively. These data showed that the fluorescent HUMARA is highly reproducible between multiple assays of the same sample and also that the results obtained by fluorescent HUMARA were equivalent to those obtained by radioactive HUMARA.

3.2.2.5 ET Clonality Comparisons

As the results from the test samples compared accurately with previous radioactive results, the HUMARA was tested for reproducibility and comparison to radioactive data in 18 ET patients. The HUMARA was carried out as described above using DNA obtained from the neutrophils (test tissue) and T-cells (control tissue) of the 18 ET patients. As for the test samples, duplicate results were obtained and the mean of the 2 results compared to radioactive data.

The mode difference between duplicates was 1%, the mean difference was 3.1% and the median difference was 2%. The percentage difference between duplicate results ranged from 0% to 22%, and the correlation coefficients were r=0.980 and r^2=0.960 (Figure 3.9A, Table 3.3). The result which had a difference of 22% between duplicates in the

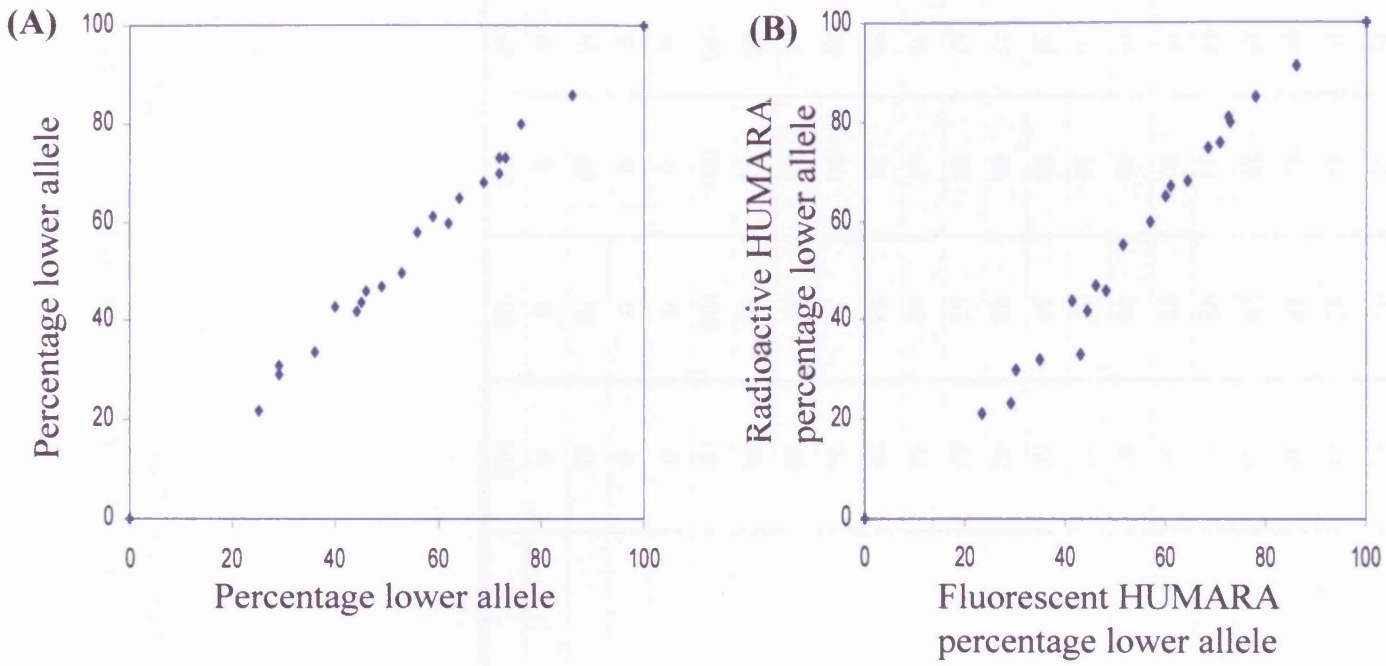

Figure 3.8
HUMARA : Graphic comparison of test sample results
(A) - comparison of the lower allele percentage for duplicate runs of each sample
(B) - comparison of fluorescent data to previously obtained radioactive data

Sample	First run % lower allele	Second run % lower allele	Fluorescent average % lower allele	Radioactive average % lower allele	Percentage difference between average fluorescent and radioactive results
1	69	68	69	75	6
2	29	31	30	30	0
3	73	73	73	80	7
4	72	73	73	81	8
5	40	43	42	44	2
6	49	47	48	46	2
7	29	29	29	23	6
8	44	42	43	33	10
9	53	50	52	55	3
10	46	46	46	47	1
11	36	34	35	32	3
12	56	58	57	60	3
13	45	44	45	42	3
14	62	60	61	67	6
15	59	61	60	65	5
16	25	22	24	21	3
17	72	70	71	76	5
18	64	65	65	68	3
19	86	86	86	91	5
20	100	100	100	100	0
21	0	0	0	0	0
22	0	0	0	0	0
23	76	80	78	85	7
24	0	0	0	0	0
25	100	100	100	100	0

Table 3.2
HUMARA assay test data
Table showing the two HUMARA results for each sample, their averages and comparitive radioactive data

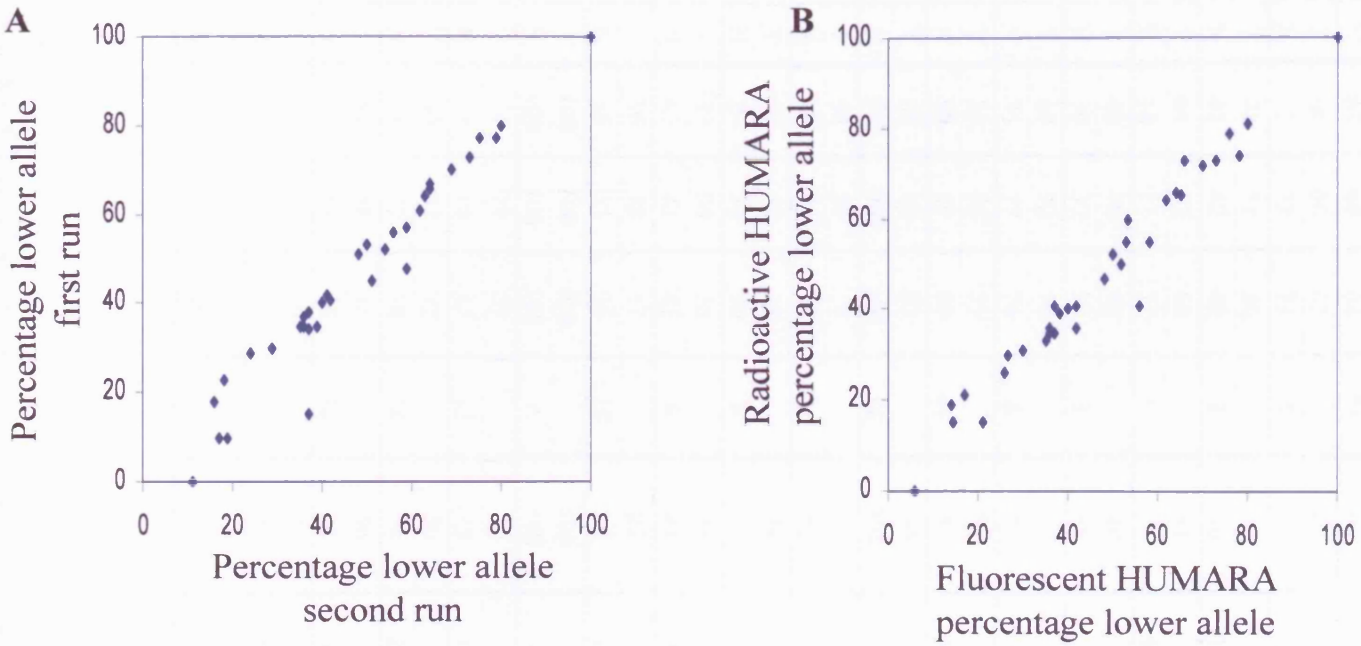

Figure 3.9
HUMARA : Graphic comparison of ET sample results
A - comparison of the lower allele percentage for duplicate results of each sample
B - comparison of fluorescent data to previously obtained radioactive data

		Fluorescent assay				Radioactive assay			
Sample Number	Cell type	First run % lower allele	Second run % lower allele	Mean % lower allele	T-N	Mean % lower allele	T-N	Difference between means	Difference between T-Ns
1	T	54	52	53		55		2	
	N	63	64	64	11	66	11	2	0
2	T	64	66	65		65		0	
	N	73	73	73	8	73	8	0	0
3	T	48	51	50		52		2	
	N	41	42	42	8	36	16	6	8
4	T	64	67	66		73		7	
	N	18	23	21	45	15	58	6	13
5	T	40	40	40		40		0	
	N	36	37	37	3	35	5	2	2
6	T	69	70	70		72		2	
	N	75	77	76	6	79	7	3	1
7	T	35	35	35		33		3	
	N	80	80	80	46	81	45	1	1
8	T	62	61	62		64		2	
	N	100	100	100	38	100	36	0	2
9	T	59	57	58		55		3	
	N	39	35	37	21	41	14	4	7
10	T	37	38	38		39		1	
	N	29	30	30	8	31	8	1	0
11	T	37	34	36		35		1	
	N	42	41	42	6	41	6	1	0
12	T	16	18	17		21		4	
	N	36	35	36	19	36	15	0	4
13	T	100	100	100		100		0	
	N	100	100	100	0	100	0	0	0
14	T	24	29	27		30		3	
	N	19	10	15	12	15	15	0	3
15	T	50	53	52		50		1	
	N	79	77	78	26	74	24	5	2
16	T	59	48	54		60		6	
	N	37	15	26	28	26	34	0	6
17	T	56	56	56		41		15	
	N	11	0	6	50	0	41	6	9
18	T	17	10	14		19		5	
	N	51	45	48	34	47	28	1	6

Table 3.3

HUMARA: Clonality test data for the ET samples

Table showing the two results for each sample and the equivalent radioactive results. T=T-cells, N=Neutrophils

T-N=Difference between T-cells and neutrophils

neutrophil fraction gave a mean neutrophil value that exactly matched the radioactive data (Table 3.3 number 16). Therefore, this sample was not investigated further. These data show that the fluorescent HUMARA is highly reproducible in ET patients. The results obtained by the fluorescent HUMARA compared well to results obtained by radioactive HUMARA. The mode difference between the fluorescent and radioactive data sets was 0%, the mean difference was 2.6%, and the median difference was 2%, range 0-15% (Figure 3.9A, Table 3.3). Values for r and r^2 were 0.987 and 0.974 respectively. Using the criteria of greater than 75% expression of one allele and more than 20% difference between T-cell and neutrophil results, twelve of the 18 ET patients tested were polyclonal by radioactive assay, 5 were clonal and 1 was uninterpretable due to an imbalanced XCIP in the T-cell fraction. All 18 ET patients tested by fluorescent HUMARA had the same clonality outcome as that obtained by the radioactive method. These data show that the mean of the duplicate results obtained by fluorescent HUMARA are equivalent to results obtained by radioactive HUMARA in ET patients.

3.2.3 IDS assay

Clonality assays which use RNA as the sample material can often be used when individuals are not informative for the DNA assays. They can also be used to confirm results obtained by DNA methods. Therefore, development of fluorescent RNA clonality assays was desirable. RNA based clonality assays use direct measurement of gene expression to establish a clonality result, and of the five RNA assays available (IDS, p55, G6PD, FHL and BTK), the IDS assay was studied because the polymorphism has a widespread distribution in the general population and the assay gave reliable, reproducible results as a radioactive assay. As all the RNA based clonality assays use the same methodology, i.e. a PCR reaction followed by a restriction enzyme digestion of the PCR product, the optimized conditions required for the IDS assay should apply to all the other RNA assays.

3.2.3.1 Screen

DNA was used to screen samples for the IDS polymorphism. Patients polymorphic at the IDS locus were identified by RFLP analysis. Thirty five cycles of PCR were carried

out with 0.5µl of DNA using primers IDS/FD and IDS/RB (Table 3.1) at an annealing temperature of 64°C with Bioline reagents. A digestion reaction containing 10µl of PCR product, 5U *BclI* and 1x buffer 3 (NEB) was incubated at 50°C for 4 hours prior to electrophoresis on 3% agarose gel to identify heterozygotes for the C to T polymorphism in codon 146 of the IDS gene. T containing alleles were digested to130bp and 30bp products. C containing alleles remained undigested at 160bp.

3.2.3.2 Radioactive assay

All radioactive clonality data presented had been previously obtained in the laboratory (Harrison *et al*, 1998a).

3.2.3.3 Fluorescent assay Optimization

Basic Conditions

As with the HUMARA assay, the initial emphasis was directed at obtaining a peak height within the limits of detection of the instrument. Only PCR cycle number was studied as appropriate injection times and loading volumes had been previously established during the optimization of the HUMARA. Neutrophil RNA from three haematologically normal control subjects was used to generate RT-PCR products. An RT reaction was set up as described in chapter 2 (2.2.6). PCR was carried out as described in chapter 2 (2.2.7) using 2µl RT reaction, at an annealing temperature of 64°C with primers IDS/FR (5' FAM fluorescent label) and IDS/RB (Table 3.1) to produce a product of 232bp. Primers were designed to so that RT-PCR product spanned an intron/exon boundary. This would enabled contaminating DNA to be detected due to its increased size compared to the RNA product. Separate PCR reactions were carried out for each sample at 20, 25 and 30 cycles. Two µl PCR product was added to 12µl de-ionized formamide and 0.5µl GS500 TAMRA size standard and loaded onto the ABI310 with an injection time of 10 seconds and a run time of 19 minutes.

The initial objective was simply to obtain a clean PCR product with a peak height within the detection limits of the instrument, so initially samples were not digested. If any problems were experienced this would help to identify the cause, i.e. whether they were related to the PCR reaction or to the digest conditions.

The results for each sample were similar and displayed the same trend of increasing peak height with increasing cycle number. A representative sample is shown in Figure 3.10. Twenty cycles produced a very small peak of approximately 200bp and nearly 200FU in height. Results were further complicated by high background and the presence of a spurious peak which was more rounded than the IDS peak, but of similar height and size, and would therefore interfere with quantification. Thirty cycles saturated the detector, the IDS peak had a flat top (indicative of saturation) and a number of smaller, less intense, spurious peaks were evident. However, 25 cycles gave a large peak of approximately 7000FU, unsaturated, but very close to saturation point. While even here there were a number of spurious peaks, they were tiny in relation the IDS peak and should not interfere with quantification. Because the effects of the restriction enzyme digestion on peak height were unknown at this point, 25 cycles were chosen as an appropriate starting point.

3.2.3.4 The effect of enzyme digestion on peak height

The effect of enzyme digestion on peak height was investigated. PCR products were generated from each of 3 neutrophil samples, as described above, using 25 amplification cycles. To an enzyme digestion reaction containing 5U *Bcl*I and 1x buffer 3 (NEB), 15µl of PCR product was used in a total volume of 18µl. The reaction was incubated at 50°C for 4 hours. T containing alleles were digested to 206bp and 30bp products. C containing alleles remained undigested at 236bp. Two µl each of the digestion reaction and the undigested PCR product were added to separate aliquots of 12µl de-ionized formamide and 0.5µl GS500 TAMRA size standard. Samples were then loaded onto the ABI310 with 10 seconds injection time and 19 minutes run time.

Digestion of the PCR product had a profound effect on the peak height (Figure 3.11 - the blue peaks are from the PCR product while the red peaks are from the GS500 TAMRA size standard used to accurately size the PCR product peaks). In spite of peak heights in excess of 3000FU for undigested PCR product, the peak height after digestion was reduced to only 600FU. There was concern that the DNA may be degrading during the enzyme reaction because the optimal temperature for *Bcl*I digestion is 50°C. Also the digestion is carried out under higher salt conditions than the PCR and this may affect the efficiency with which DNA is electrophoresed onto the capillary. The final salt

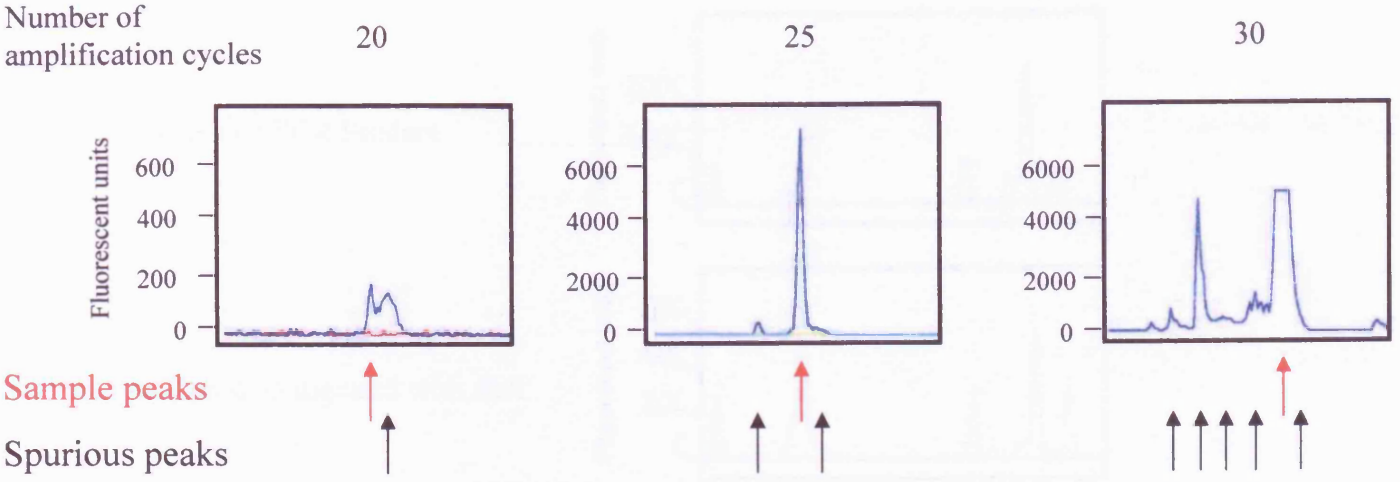

Figure 3.10
IDS : The effect of increasing PCR cycles on peak height
Black arrows indicate spurious peaks which affect quantification when peak height is low, but are negligible with higher peaks. Correct IDS peak at 236bp is indicated by a red arrow.

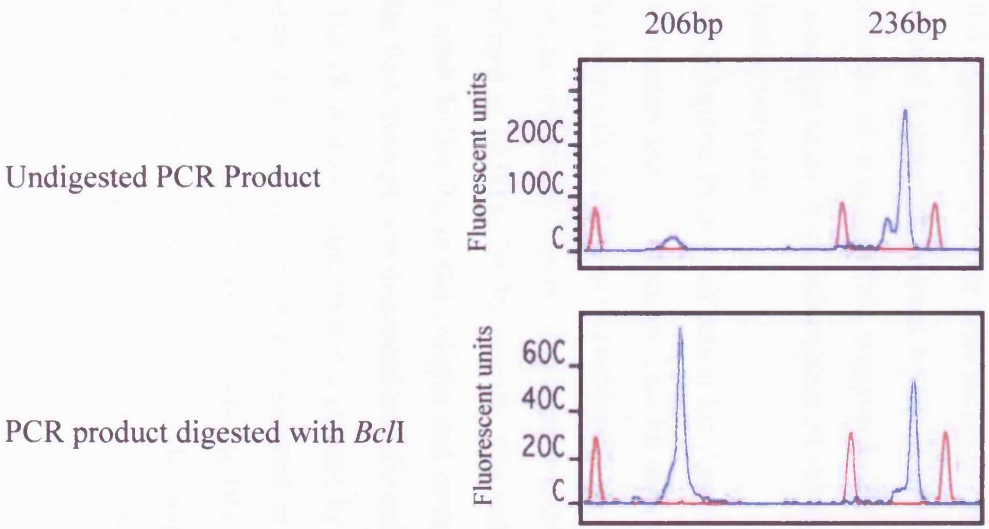

Figure 3.11

IDS : Effect of *BclI* digestion on the peak height of fluorescently labelled IDS PCR product

GS500 size standard peaks are shown in red, sample peaks are blue. A spurious peak is visible in the untreated PCR product at around 200bp.

concentrations for the enzyme digestion are 100mM NaCl and 10mM $MgCl_2$ compared to the salt in the PCR reaction of 16mM $(NH_4)_2SO_4$ and 1mM $MgCl_2$.

To test the effect of the high incubation temperature and salt concentration on peak height, one neutrophil sample was treated in six different ways prior to loading onto the instrument: (a) PCR product digested for 1 hour, (b) PCR product digested for 2 hours, (c) PCR product digested for 4 hours, (d) PCR product, (e) PCR product incubated at 50°C for 4 hours and (f) PCR product incubated under digestion reaction conditions for 4 hours, but in the absence of enzyme (Figure 3.12). The PCR product (d) had a peak height of over 3000FU. Incubation of the PCR product at 50°C for 4 hours (e) had no effect on peak height. All 4 samples (a-c, f) that were incubated at the high salt conditions of the digestion reaction showed a reduction in peak height to less than 1000FU, irrespective of incubation time or presence of enzyme. This suggested that the reduction in peak height following digestion was due to the high salt content of the digest reaction conditions rather than the high incubation temperature.

The QIAquick PCR purification kit (Qiagen) was used to remove the excess salt from the restriction enzyme reaction. This kit uses silica-membrane spin columns that bind DNA under high salt and low pH conditions. Five volumes of the binding buffer PB were mixed with the restriction enzyme digest prior to loading onto a spin column. The column was centrifuged at 18,000g for 30 seconds. The columns were then washed by addition of 0.75ml of wash buffer PE to the column and centrifuged at 18,000g for 30 seconds. The wash buffer flow through was discarded and the column centrifuged for a further minute at 18,000g. The DNA was eluted from the column by addition of 30µl of water to the centre of the membrane. The column was incubated at room temperature for 1 minute, and centrifuged for 1 minute at 18,000g to elute the DNA.

One neutrophil sample and one T-cell sample were used to evaluate the clean-up system. PCR products were generated using 25 cycles of amplification and digested as as previously described. A 2µl aliquot of the digestion product was loaded onto the instrument without any further treatment. The remaining digest reaction was cleaned using the QIAquick PCR purification kit prior to loading. The product peaks from un-cleaned aliquots were approximately 200FU and 700FU for T-cells and neutrophils respectively (Figure 3.13). A number of spurious peaks were also evident, some of which may interfere with quantification. However, use of the clean-up prevented the reduction in peak height

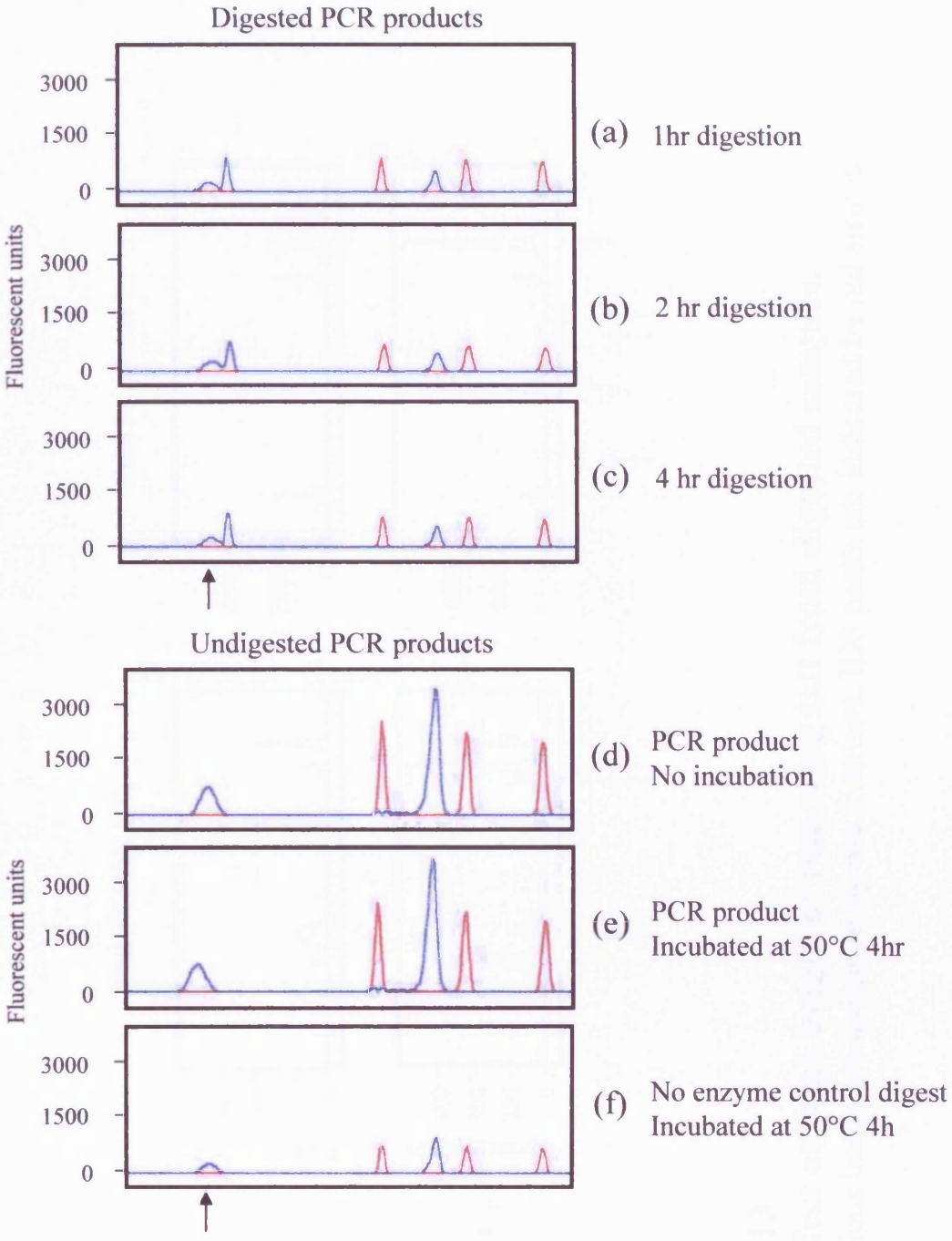

Figure 3.12
IDS : Effect of *BclI* digestion conditions on peak height.
A spurious peak is visible in both the digested and undigested PCR products (indicated by arrow).

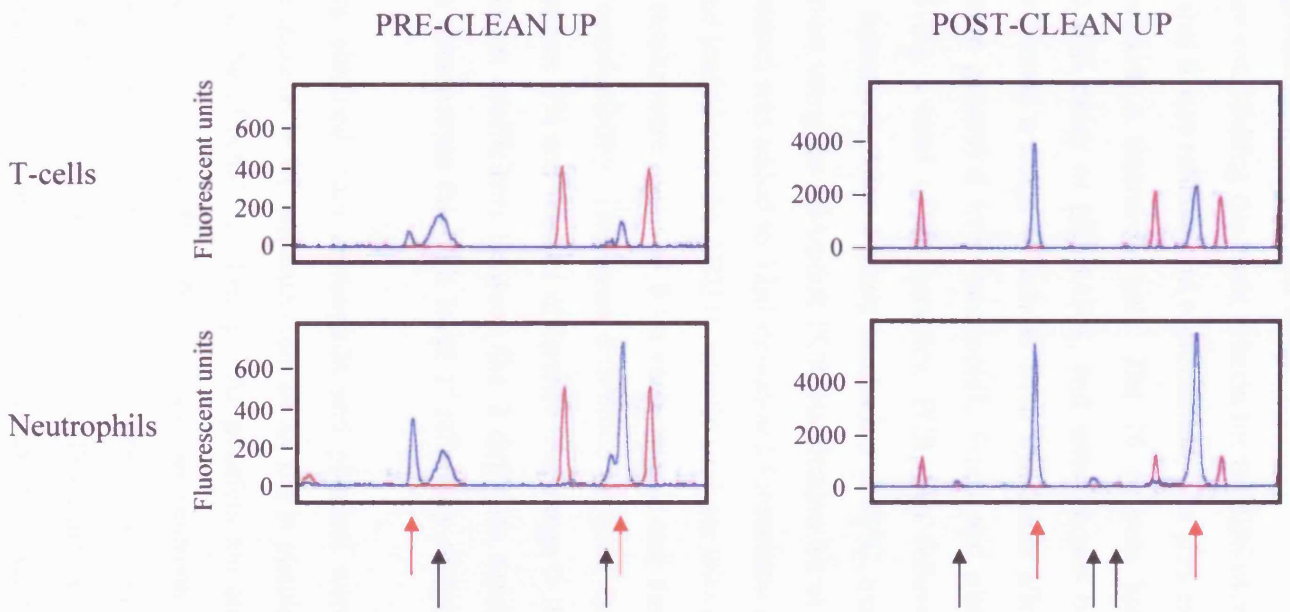

Figure 3.13

IDS : Effect on peak height of removing salt from digested samples.

Large spurious bands are indicated by black arrows, IDS peaks are indicated by red arrows

seen after digestion. The 2 samples that had been cleaned both had highest peak heights in excess of 4000FU, and while spurious peaks were still present, they were small and unlikely to interfere with quantification.

3.2.3.5 Reproducibility of the IDS assay

After establishing the basic criteria for the IDS clonality assay, it was important to determine that it was reliable and reproducible. The IDS assay was carried out on a set of 10 patients with a thrombocytosis. The 10 patients had previously been studied by radioactive IDS assay or HUMARA, and were chosen because their previous clonality results represented a range of results from 0% lower allele to 100% lower allele. PCR products were generated from neutrophil, T-cell and platelet RNA for each of the 10 patients giving a total of 30 samples. PCR was followed by digestion with *Bcl*I as previously described. After 4 hours incubation at 50°C, excess salt was removed from the digest reaction using the QIAquick PCR purification kit as described above. A 2µl aliquot of PCR product was added to 12µl de-ionized formamide and 0.5µl GS500 TAMRA size standard and loaded onto the ABI310 genetic analyser with an injection time of 10 seconds. Duplicate results were obtained from each sample and the two results were compared to establish reproducibility. The mean difference between duplicate results was 2.4%, with mode difference 2% and median difference 2%, range 0-10% (Figure 3.14 A, Table 3.4). The correlation coefficients between the 2 duplicate results were $r=0.994$ and $r^2=0.989$. These data demonstrate the high level of reliability of the fluorescent IDS for these test samples.

Data obtained from neutrophils and platelets were also compared. As the MK lineage is expanded in ET, measurement of XCIPs in platelets provides a direct measure of the XCIP of the affected cell lineage. As platelets are anucleate, only RNA assays can directly measure their XCIP. DNA assays use neutrophils to measure clonality of the myeloid cell lineage as neutrophils and MKs are derived from a common myeloid progenitor. RNA assays can be used to establish whether neutrophils are a valid tissue to use to measure clonality in ET, as a comparison between XCIP results for neutrophils and platelets can be made. The mean difference between neutrophil and platelet XCIP results for the 10 patients was 3.7%, with mode difference 4% and median difference 3.5%, range 0-10% (Figure 3.14 B, Table 3.4). Values for r and r^2 were 0.991 and 0.983 respectively. This data suggests that the XCIP of the neutrophil sample is representative of the XCIP in

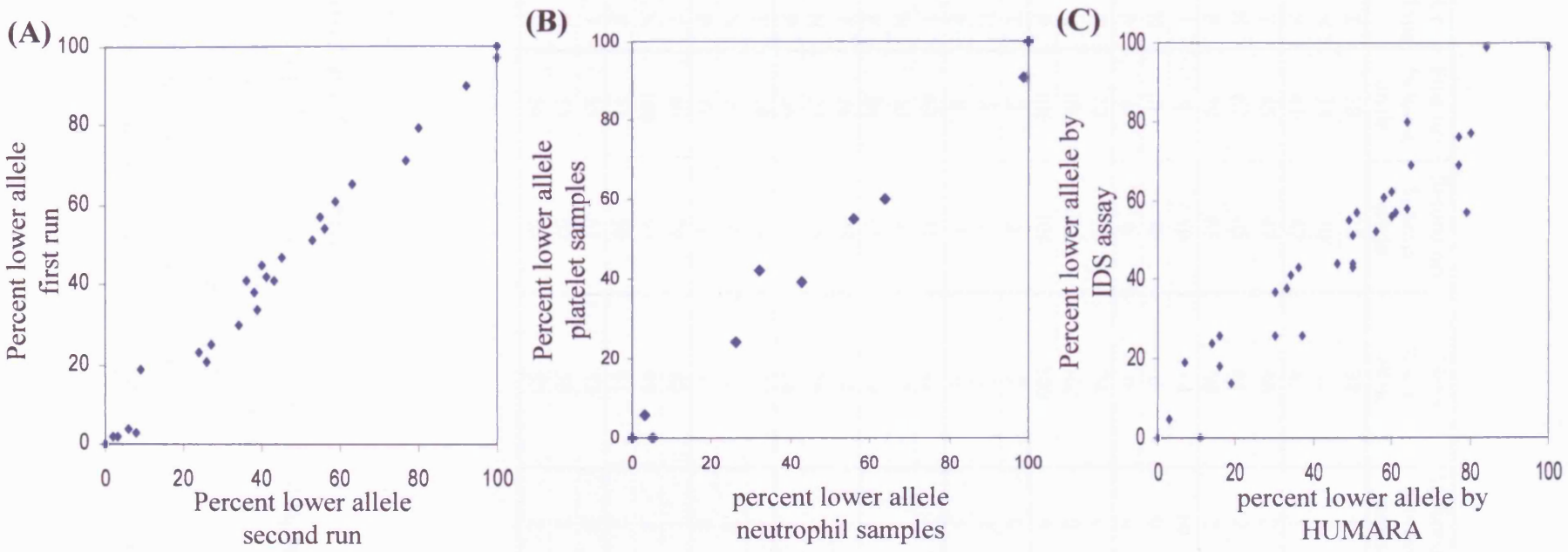

Figure 3.14
IDS Comparison Graphs

(A) - comparison of the lower allele percentage for duplicates of each sample
(B) - comparison of results obtained for neutrophils and platelets
(C) - comparison of results obtained by DNA HUMARA and RNA IDS for each sample

Sample	Cell Type	First run % lower allele	Second run % lower allele	Mean % lower allele	Difference between means	T-N	T-P	Difference between T-N and T-P
1	T	38	38	38	0			
	N	34	30	32	4	6		2
	P	41	42	42	1		4	
2	T	45	47	46	2			
	N	63	65	64	2	18		
	P	59	61	60	2		14	4
3	T	9	19	14	10			
	N	0	0	0	0	14		
	P	0	0	0	0		14	0
4	T	77	71	74	6			
	N	100	100	100	0	26		
	P	100	100	100	0		26	0
5	T	2	2	2	0			
	N	3	2	3	1	1		
	P	8	3	6	5		4	3
6	T	43	41	42	2			
	N	40	45	43	5	1		
	P	36	41	39	5		3	2
7	T	24	23	24	1			
	N	27	25	26	2	2		
	P	26	21	24	5		0	2
8	T	39	34	37	5			
	N	6	4	5	2	32		
	P	0	0	0	0		37	5
9	T	80	79	80	1			
	N	100	97	99	3	19		
	P	92	90	91	2		11	8
10	T	53	51	52	2			
	N	55	57	56	2	4		
	P	56	54	55	2		3	1

Table 3.4
IDS clonality test data

Table showing the two IDS results for each sample, the average result and the difference between the two results

T=T-cells N=neutrophils P=platelets

T-N=Difference between T-cell and neutrophil results

T-P=Difference between T-cell and platelet results

platelets for patients with a thrombocytosis, giving validity to the assumption that neutrophils can be used as a measure of XCIP in the megakaryocytic compartment in ET patients.

3.2.4 Comparison of DNA and RNA clonality results

DNA and RNA clonality assays should give equivalent results. The average of duplicate HUMARA and IDS assay results were examined to ensure this was the case. The results from a total of 18 individuals were used. Of the 18 individuals 9 were ET patients, 6 were normal controls and 3 were patients with a reactive thrombocytosis.

HUMARA and IDS assay results from 9 ET patients were obtained, generating 17 comparisons from 8 T-cell results and 9 neutrophil results. The mean difference between HUMARA and IDS assay results for ET patients was 7.2%, and the median difference was 6%, range 0-22% (Figure 3.14 C, Table 3.4). Values for r and r^2 were 0.95 and 0.90 respectively. Of the 9 patients studied 2 were clonal, 4 were polyclonal and 2 were uninterpretable by both HUMARA and IDS assay. The remaining patient could only be analysed by neutrophils at both RNA and DNA levels and thus clonality could not be established by either HUMARA or IDS assay due to the lack of control tissue results. However, this patient had only a 1% difference between HUMARA and IDS assay results. In this cohort of nine patients, DNA results by HUMARA are equivalent to RNA results by IDS assay.

HUMARA and IDS assay results from 6 haematologically normal control subjects were obtained, generating 12 results for comparison, 6 from T-cells and 6 from neutrophils. The mean difference between DNA and RNA results was 3.5% and the median difference was 4.8%, range 1-11%. Correlation coefficients were r=0.96 and r^2=0.91. All normal controls were either polyclonal or uninterpretable due to an imbalance XCIP in both T-cells and neutrophils, by both HUMARA and IDS assay.

Overall 16 T-cell results and 18 neutrophil results were obtained from both HUMARA and IDS assay for the 18 individuals studied. The mean difference between DNA and RNA results was 6.3% and the median difference was 5.5%, range 0-22%. Correlation coefficients were r=0.95 and r^2=0.90.

These data confirm the accuracy of results obtained by both the HUMARA and IDS assays as differences between XCIP results obtained from them are low, the two sets of

results have correlation coefficients greater than 0.9, and both assays give the same clonality outcome for both patients and normal controls.

3.3 Discussion

The use of fluorescently labelled PCR primers has become feasible in the research laboratory due to their wide availability and falling costs. They have begun to replace radioactive isotopes as they have a number of advantages over them. However, to replace radioactive isotopes in established clonality assays it was important to ensure that reliable, reproducible results could be achieved using fluorescent primers and an automated genetic analyser. By establishing appropriate conditions for fluorescent clonality assays, and by comparison of clonality data obtained by fluorescent technology with previously obtained data using radioactive technology, this could be determined.

HUMARA assay

The basic conditions for the use of HUMARA were established as 22 PCR cycles with a 15 second injection time and a PCR product volume of 2μl. These conditions gave a peak height within the detection limits of the ABI310 genetic analyser, and results that were reliable and reproducible

IDS assay

The basic conditions of the IDS assay were established as 25 PCR cycles with 15 seconds injection time and a sample volume of 2μl. While restriction enzyme digestion reduced the peak height to very low levels, this was shown to be due to the high concentration of salt in the digest reaction. Peak height equivalent to the original PCR products was restored after removal of the excess ions with a PCR clean-up kit. The negatively charged ions in the digest reaction are a problem because they carry the same charge as DNA, but as they are much smaller molecules than the DNA of the digest product, they are more readily electrophoresed onto the capillary. Thus, although the amount of digest product in the loading solution may be high, it is mainly the cationic salts that are loaded onto the capillary during the injection of the sample. As the salt concentration of a PCR reaction is lower than in an enzyme digest, this problem was not so

evident with the HUMARA. Once a clean up protocol was used, results became reliable and reproducible.

A good correlation could also be demonstrated between RNA and DNA results obtained by fluorescent and radioactive techniques. Results obtained from 36 samples, assayed by both HUMARA and IDS, showed a good correlation with $r=0.950$ and $r^2=0.903$. The clonality results were the same using either RNA or DNA based assays, and whether using neutrophils or platelets as the test tissue.

As the peak height between different samples is variable depending on the starting amount of DNA or RNA, it was important to define a set of criteria that needed to be met before a clonality result could be accepted. After gaining some experience with the technique it was decided that the following criteria must be met in order for the clonality result to be accepted.

1: The height of the highest peak must be greater than 1,000FU

2: The overall clonality result should be based on the average of two results, and as result variability can be as high as 20%, the duplicate results should be within 10% of one another.

3: If the first criteria is not met, then the result can be accepted if one result has a highest peak height of over 1,000FU, and the second result is within 10% of the first, without the presence of spurious peaks which may interfere with quantification.

Accurate, reproducible quantitative fluorescently labelled HUMARA and IDS assays can be carried out by following the above criteria. Results which are equivalent to the previously used radioactive technique can be obtained, but without the problems associated with the handling of radioactive isotopes. All the other PCR based clonality assays in common usage can also be carried out using the techniques described above, but with the PCR primers and restriction enzymes appropriate to that assay.

Chapter 4

Clonality in Essential Thrombocythaemia

4.1 Introduction

4.1.1 Clonality in ET

The World Health Organization describes ET as a clonal disorder, caused by one or more unknown, acquired alterations in a haemopoietic stem cell leading to an expansion of the MK compartment (Pierre *et al*, 2001). This view stems from the first clonality study of ET patients using the G6PD isozyme assay (Fialkow *et al*, 1981). As no molecular markers have yet been identified for ET and the proportion of patients with cytogenetic abnormalities is small, clonality in ET patients cannot be evaluated directly. However, clonality can be assessed in female ET patients using XCIPs. In the Fialkow study, both the A and B form of G6PD were present in non-haemopoietic tissue obtained from two patients with a diagnosis of ET, and one patient with a probable diagnosis of ET. However, only one isoform could be found in the MK, red cell and granulocytic cell lineages, indicating that ET was clonal and arose from a multipotent haemopoietic stem cell. Another study used Epstein-Barr Virus-transformed lymphoblastoid cell lines derived from one ET patient to show a clonal pattern of cell growth (Raskind *et al*, 1985); 104 of 109 EBV cell lines monotypic for G6PD isozyme carried the enzyme type of the abnormal stem cell clone, indicating a largely clonal cell population. A third study used PGK and HPRT Southern blot assays to study clonality in ET (Anger *et al*, 1990). Three out of four patients displayed a predominantly clonal pattern in peripheral blood samples. One of the three was further investigated and demonstrated a clonal granulocyte fraction and a non-clonal T-lymphocyte fraction.

The studies described above are limited in their usefulness. They used small numbers of patients (just 8 patients in total from the three studies), did not exclude patients due to age, and either used none or inappropriate control and/or test material. As described in chapter 3 (3.1.6) a number of exclusion criteria must be applied to XCIP assays. T-cell control tissue must be used as a reference to control for a constitutively imbalanced XCIP, and the difference between T-cell and neutrophil results should be greater than 20%. Also, samples should be from individuals less than 65 years of age to eliminate age related skewing. As more groups investigated clonality in larger ET cohorts and used appropriate exclusion criteria, it became obvious that a proportion of ET patients do not have a clonal

disorder. The results of these studies are summarized in Table 4.1. The earliest of these studies showed that a proportion of female ET patients interpretable by XCIP analysis (10/29, 34%) appeared not to have clonal myelopoiesis (el Kassar *et al*, 1997). Another study showed that in 46 patients only 23 were interpretable by XCIP, and of those 10 (43%) were clonal and 13 (57%) were polyclonal by DNA and RNA XCIP analysis (Harrison *et al*, 1999a). This data was confirmed by other groups with the percentage of clonal patients within the ET cohorts varying from 43% to 67% (Liu *et al*, 2003; Teofili *et al*, 2002a). The largest study investigated clonality in 88 interpretable ET patients, 53 (60%) patients had clonal myelopoiesis and 35 (40%) had polyclonal myelopoiesis (Vannucchi *et al*, 2004). When taken together, these studies clearly demonstrate that ET patients can be divided into two groups based on clonality status, those with a clonal disorder and those with a polyclonal disorder. Of the 245 ET patients studied in total, 146 (60%) had clonal myelopoiesis and 99 (40%) had polyclonal myelopoiesis.

4.1.2 Significance of Clonality Status in ET

To further characterize the 2 groups of ET patients, a number of studies have compared clonality with the clinical features associated with ET. In a study of 46 ET patients, 23 were un-interpretable due to a constitutively imbalanced XCIP in T-cell control tissue or because they had a clonal XCIP but were greater than 65 years of age. Of the remaining 23, 10 were clonal and 13 were polyclonal by DNA and RNA XCIP analysis (Harrison *et al*, 1999a). No difference between the clonal or polyclonal groups could be found in age, platelet count at diagnosis, incidence of hepatomegaly or splenomegaly, or haemorrhagic complications. However, there was a significant association between clonal myelopoiesis and the incidence of thrombosis. Six of the 10 clonal patients (60%) had had thrombotic events compared to 2/13 (15%) polyclonal patients ($p<0.039$).

Three other groups have reproduced this data. The first of these described a cohort of 40 ET patients in which 32 were informative and interpretable for DNA-based HUMARA XCIP analysis or RNA-based IDS or p55 analysis (Chiusolo *et al*, 2001). A significant correlation was found between clonal myelopoiesis and an increase in thrombotic risk, 7 (41%) out of 17 clonal patients had had a thrombotic event, compared to

Study	n*	Clonal Patients	Polyclonal Patients
(el Kassar *et al*, 1997) #	29	19 (66%)	10 (34%)
(Harrison *et al*, 1999a)	23	10 (43%)	13 (57%)
(Ferraris *et al*, 1999)+	13	6 (46%)	7 (54%)
(Chiusolo *et al*, 2001)	32	17 (53%)	15 (47%)
(Shih *et al*, 2002)	48	33 (69%)	15 (31%)
(Teofili *et al*, 2002a)	12	8 (67%)	4 (33%)
(Liu *et al*, 2003)++	12	8 (67%)	4 (33%)
(Vannucchi *et al*, 2004)	88	53 (60%)	35 (40%)
Total	245	146 (60%)	99 (40%)

Table 4.1

Clonality studies in ET patients.

* Number of patients interpretable by XCIP analysis

No exclusion due to age

+ No exclusion due to age or a constitutively imbalanced XCIP

++SSCP transcriptional clonality assay

1 (6.7%) of the 15 polyclonal patients (p=0.04). No correlation was found between clonality results and age, platelet count at diagnosis, or EEC positivity. In the large cohort of a second group, 17/53 (31%) clonal patients had had a thrombotic complication compared to 2/35 (6%) polyclonal patients (p<0.009) (Vannucchi *et al*, 2004). In this cohort EEC positivity, increased PRV-1 expression and c-mpl mRNA expression levels were also investigated with respect to incidence of thrombosis, but no correlation between thrombotic events and any of these markers could be found. Results from a third group did not reach statistical significance, with 11/33 (33%) clonal ET patients and 2/15 (13%) polyclonal patients having had a thrombotic event (p=0.073), but the results did support the trend towards clonal patients being at higher risk of thrombotic events (Shih *et al*, 2002).

4.1.3 Clonality status and disease progression

Why ET patients can have either a clonal or polyclonal disorder, but still present with similar symptoms is unclear. Clonality may define two distinct ET subgroups which present with similar symptoms, but have different underlying defects and are phenotypically distinct, at least in relation to thrombotic risk. Clonal ET may represent a low grade malignant disorder and polyclonal ET a dysregulation of platelet production due to either extracellular stimulatory facts such as raised levels of cytokines, or an intrinsic hyper-responsiveness to such stimuli. However, it is unknown whether a polyclonal disorder can progress to a clonal disorder.

Polyclonal ET may represent an initial phase of ET during which there is excessive external drive to megakaryocytopoiesis. Stem cell mutations may then arise which impart a growth or survival advantage leading to a clonal proliferation. Secondly, polyclonal ET could represent a situation where a hyperproliferating clone is contributory to the platelet and myeloid production but is not yet sufficient for the whole population to be deemed clonal by XCIP analysis, for this to be true the normal platelet production would not have to be subject to end cell negative feedback control. Thirdly, it is possible that a stem cell defect could give rise to variable numbers of mature end cells in the different lineages. Thus it is conceivable that a mutated stem cell could contribute to nearly all the platelet pool without contributing greatly to the neutrophil pool, these being largely derived from residual normal stem cells

4.1.4 Technical considerations

In spite of the large number of studies describing polyclonal ET there is still controversy concerning the high proportion of polyclonal patients, and some questions remain over technical aspects of the assays. Hypermethylation is a common feature of neoplasia, and has often been reported in gene promoter regions, where it can suppress gene transcription (Herman & Baylin, 2003). It has also been shown to occur in some genes from patients with each of the cMPDs. The promoter region of the calcitonin gene has been shown to be highly hypermethylated in cIMF, and to a lesser degree in PV and ET (Ihalainen *et al*, 1994). In CML the promoter region of JunB was hypermethylated in all 32 patients studied, with no hypermethylation evident in 17 controls (Yang *et al*, 2003). More recently, the RARβ2 gene promoter was shown to be hypermethylated in CD34+ cells from 89% (16/18) of cIMF patients compared to methylation levels observed in any of the 9 control CD34+ samples (Jones *et al*, 2004). These studies suggest that hypermethylation could occur in ET and therefore may affect results of clonality studies which exclusively use DNA based assays.

The majority of ET clonality publications have made use of the DNA-based HUMARA which relies on digestion of unmethylated DNA to distinguish between the active and inactive X-chromosomes. Technical problems may arise if hypermethylation occurs at the CpG island in the *Hpa*II recognition sequence of the HUMARA PCR product. Methylation patterns can be heterogeneous and it has been shown that one of the *Hpa*II sites at the DXS255 locus, identified by the M27β probe, can be variably methylated. In 37 haematologically normal females there was a mean of only 67% digestion at the DXS255 locus using *Hpa*II, but digestion with a second methylation-sensitive restriction enzyme, *Hha*I, with different cutting sites at the same locus, had a mean 95% digestion (Gale & Linch, 1994b). However, in this case the variable methylation did not affect the clonality results as the correlation coefficient between XCIP results using *Hpa*II and *Hha*I digestion was 0.97, suggesting that methylation occurred at the same rate on each allele. In an extreme situation of hypermethylation, some of the active alleles from patients with a completely monoclonal XCIP would become methylated, and would not be digested by methylation-sensitive enzymes. This could lead to the XCIP from a patient with clonal myelopoiesis having a balanced XCIP, as PCR product would be generated from alleles methylated due to hypermethylation as well as from alleles methylated due to X-chromosome inactivation (Figure 4.1). This would only affect results obtained using DNA

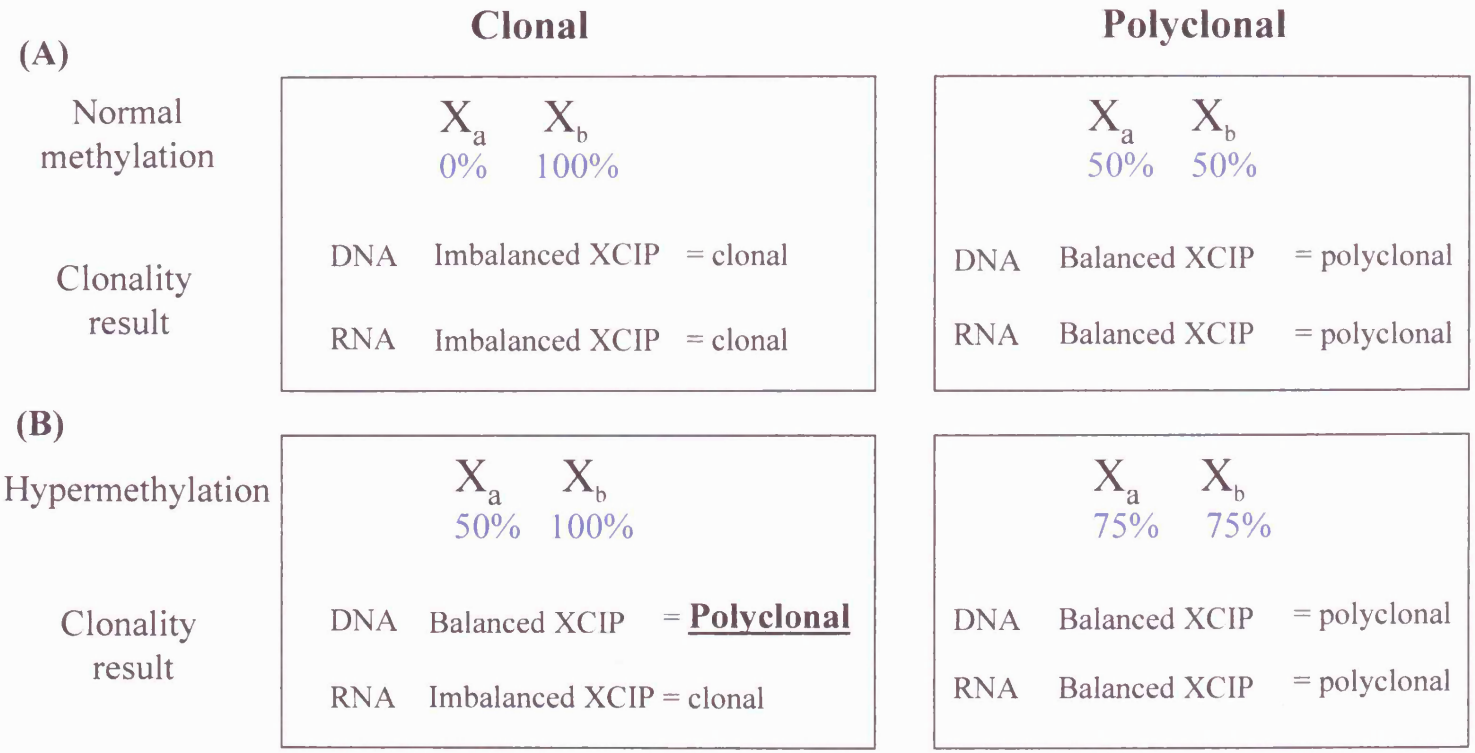

Figure 4.1

The effect of hypermethylation on clonality results using DNA and RNA based assays

(A) – The results obtained from patients with normal methylation by DNA and RNA clonality assays

(B) – The results obtained from patients with extreme hypermethylation by DNA and RNA clonality assays

Percentages in blue represent the percentage of methylated X-chromosomes. X_a and X_b represent maternal and paternally inherited X-chromosomes.

assays, where the amount of inactive (methylated) gene is measured. RNA assays that measure active (expressed) genes may see a decrease in expression levels, but the relative expression of each allele should remain the same assuming that hypermethylation occurs at the same rate on each allele.

4.1.5 Aim

The aim of the work presented in this chapter was to investigate whether clonality status changes during the clinical course of ET and whether hypermethylation of the human androgen receptor locus is a feature of ET.

4.2 Materials and Methods

4.2.1 Patients and Samples

Peripheral blood samples were obtained from 111 individuals, 100 from females and 11 from males. Of the 100 female samples, 92 had a persistent thrombocytosis and 8 were haematologically normal controls. The 11 male samples had a diagnosis of ET. DNA and RNA were prepared from purified $CD3^+$ cells and neutrophils, as outlined in Chapter 2 (2.2.1/2/3).

4.2.2 Heterozygosity at clonality assay loci

Neutrophil DNA from 100 females was screened for heterozygosity of the following genes; PGK, IDS, p55, G6PD, BTK and FHL using PCR as outlined in Chapter 2 (2.2.7). Bioline reagents were used with 35 amplification cycles and the appropriate primers and annealing temperatures for each assay as detailed in Table 4.2. Ten µl PCR product was digested with appropriate enzyme and buffer, as shown in Table 4.3, in a total volume of 12µl. Digestion reactions were incubated for at least 4 hours at the manufacturer's recommended temperature. Samples were then electrophoresed through 3% agarose gels except for samples prepared for the PGK screen, where 1.5% agarose gels were used. The expected product sizes after digestion are shown in Figure 4.2. As heterozygosity at the human androgen receptor locus is over 90% (Edwards *et al*, 1992),

Assay	Forward primer (5'→3')	Reverse primer (5'→3')	Fragment Size (bp)	Annealing Temp (°C)
HUMARA	TCCAGAATCTGTTCCAGAGCGTGC	GCTGTGAAGGTTGCTGTTCCTCAT	Variable	68
PGK	AGCTGGACGTTAAAGGGAAG	TGTGAGATGTGGGCCGTTTC	474	60
p55 (D)	CTCCTCAAAGCAGGCTTCATGCCTG	CGTACAGGACTGTTTTTCATTCAGCTTCCG†	191	64
p55 (R)	CACAGAAGAGCCCATGGGAATCGC†	CGCCTTCTGCAGCTGATCCAC	178	64
IDS (D)	GCCCCAAAGAAGGGAGGGTCC	TGGAAAAGACCAGCTATACGGAGAATGATC†	160	64
IDS (R)	TTTGCGCAGCAAGCAGTGTGCG	TGGAAAAGACCAGCTATACGGAGAATGATC†	236	64
G6PD (D)	ATACAAGAACGTGAAGCTCCCTGACGCGTA†	GTGGGTGCCCAGGGCTCAGAG	480	66
G6PD (R)	ATACAAGAACGTGAAGCTCCCTGACGCGTA†	GTGGGTGCCCAGGGCTCAGAG	281	66
FHL-1	ATGTTCCTGCAGTGCTGAAATTCATCCTCC†	CAGAAGGGACTTTGCAGTCCTC	189	62
BTK (D)	TAGGAAGACTAGGACCCCTGC	AGATCCTAATAAAGCACTTACCTCATGCTA†	247	62
BTK (R)	GCTCAGTAGGCTCCAAATTTG	AGTGGGACGCTCATCTGCTTTCTCATGCTA†	271	62

Table 4.2
PCR primers for each of the heterozygosity screens and clonality assays

Expected fragment sizes and appropriate annealing temperatures are shown for each primer pair

D = Primers for heterozygosity screen R = Primers for RNA clonality assays

FHL-1 and PGK primer pairs were used for both the heterozygosity screen and clonality assay. HUMARA primers were only used for HUMARA clonality assay.

† Mismatch primers with mismatched bases underlined

Assay	Primers	Annealing temperature (°C)	Number of amplification Cycles	Restriction enzyme (NEB)	Buffer used for restriction enzyme digestion (NEB)	Incubation temperature (°C)
p55	p55 (R) forward and reverse	64	28	*BstU*I	2	60
G6PD	G6PD (R) forward and reverse	66	28	*Rsa*I	1	37
BTK	BTK (R) forward and reverse	62	29	*Nhe*I	2 + BSA	37
FHL	FHL (R) forward and reverse	62	32	*Msp*I	2	37
IDS	IDS (R) forward and reverse	64	26	*Bcl*I	3	50

Table 4.3

Conditions used for the RNA clonality assays

Table showing the basic conditions for the PCR reactions and restriction enzyme digests used in each of the five RNA based clonality assays. Primer sequences are detailed in Table 4.2. R = Primers for RNA clonality assay NEB – New England Biolabs.

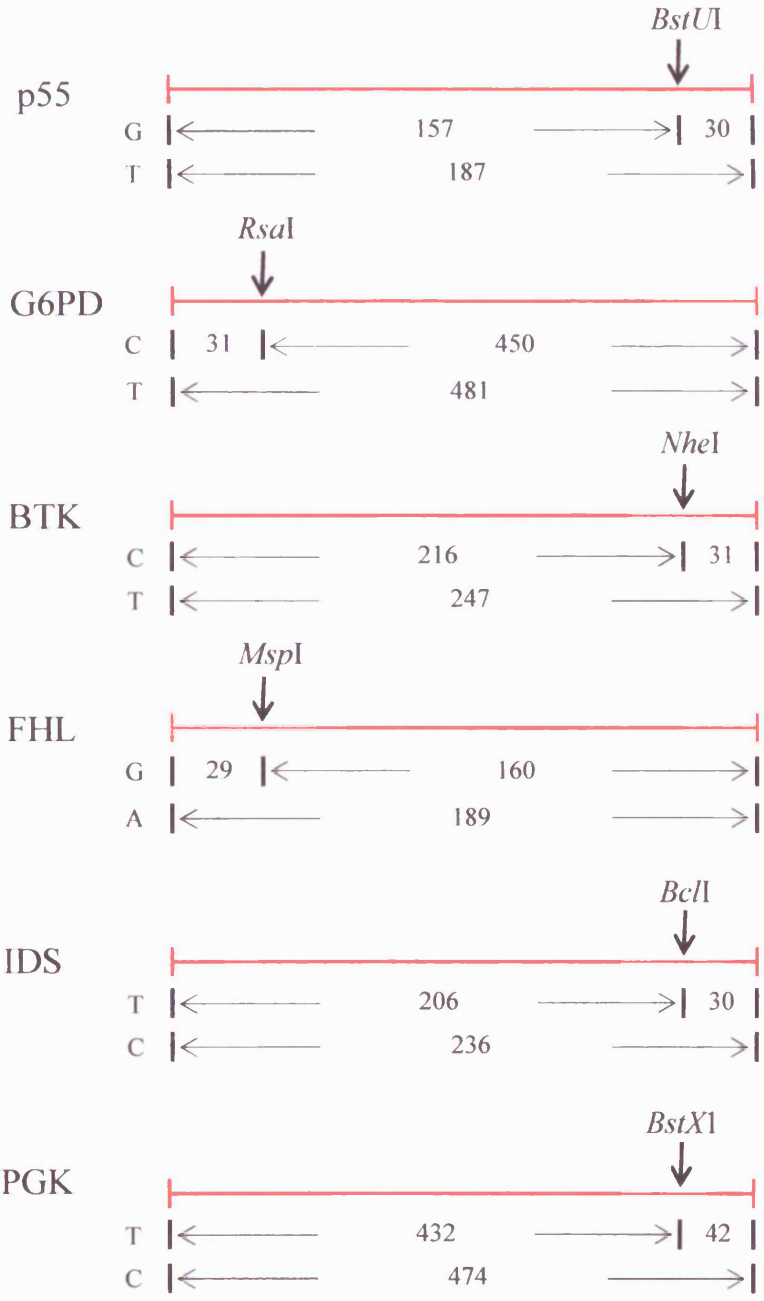

Figure 4.2
The restriction enzyme digestions used to screen for heterozygosity at 6 of the clonality assay loci
Diagram showing the enzymes required and digestion product sizes (bp) for each RNA clonality assay screen from DNA template. PCR product shown in red, products of enzyme digestion shown in black.

samples were not separately screened for this polymorphism. However, heterozygous patients could be identified during the HUMARA from the aliquot containing *Rsa*I only.

4.2.3 HUMARA assay

DNA clonality results were compared using 3 technologies, radio-labelling of primers and acrylamide gel electrophoresis, fluorescent labelling with visualization using an ABI Prism™ 310 genetic analyser (ABI310), or fluorescent labelling with visualization using a CEQ™ 8000 genetic analysis system (CEQ8000) (Beckman Coulter). All radio-labelled HUMARA data had been previously obtained in the laboratory.

HUMARA clonality data was obtained on 4 aliquots from each patient. Neutrophil and T-cell DNA were digested as outlined in chapter 3 (3.2.2.3) with either *Rsa*I alone or both *Rsa*I and *Hpa*II. Twenty two cycles of PCR were then carried out on each aliquot, using either a 5' FAM labelled HUM/F primer for the ABI310 or a DP4 labelled HUM/F primer for the CEQ8000, and the unlabelled HUM/R primer (Table 4.2) at an annealing temperature of 68°C.

Samples to be analysed on the ABI310 were prepared by adding 2µl PCR product to 12µl of de-ionized formamide and 0.5µl GS500 size standard. Samples were incubated at 95°C for 5 minutes and quenched on ice. The denatured product was then loaded onto the ABI310 genetic analyzer with an injection time of 10 seconds, and electrophoresed at 15 kV for 24 minutes. These conditions were determined by the optimization carried out in chapter 3. Samples to be analysed on the CEQ8000 were prepared by adding 2µl of PCR product to 40µl of sample loading solution (SLS) containing 0.5µl of 400PA size standard. Samples were run using fragment analysis program Frag 3. Results were analyzed as outlined in chapter 3 (3.2.1) and reported as the percentage expression of each allele, with the smaller allele quoted first. Each sample was analyzed in duplicate and the results expressed as the mean of the two duplicates.

4.2.4 RNA assays

A reverse transcription reaction was carried out as described in chapter 2 (2.2.6) using approximately 1µg of RNA from T-cells, neutrophils or platelets from each informative ET patient. PCR was then carried out as described in chapter 2 (2.2.7), with Bioline reagents. The primers used for each assay are given in Tables 4.2 and 4.3. The non

mis-match primer was labelled with either 5'FAM fluorescent label for use with the ABI310 or DP4 label for use with the CEQ8000, and PCR carried out using the appropriate number of cycles and annealing temperature detailed in Table 4.3. Samples were incubated at 95°C for 5 minutes prior to adding the Taq polymerase to ensure template cDNA was completely denatured before carrying out amplification. Ten µl PCR product was added to a reaction mixture containing 5U of restriction enzyme and 1µl of appropriate buffer (with 0.1µg/µl BSA if required) (Table 4.3). Each reaction was incubated for at least 4 hours at manufacturer's recommended temperature (Table 4.3). The results were visualized using either the ABI310 or CEQ8000 genetic analyser.

Samples prepared for analysis on the ABI310 required the removal of excess salt from the restriction enzyme reaction using the QIAquick PCR purification kit (Qiagen) as described in chapter 3 (3.2.3.4). After clean up, 2 µl sample was mixed with 12µl de-ionized formamide and 0.5µl GS500 size standard, incubated at 95°C for 5 minutes and quenched on ice. The denatured product was loaded onto the ABI310 with an injection time of 10 seconds, and electrophoresed at 15kV for 20 minutes. When using the CEQ8000, digestion products were diluted 1 part PCR product to 2 parts SLS to reduce the salt concentration, and 2µl diluted product added to 40µl SLS containing 0.5µl 400PA size standard. The mix was then run on the CEQ8000 using fragment analysis program Frag 3 and results reported as the percentage expression of the lower allele. Each sample was analyzed in duplicate and the results expressed as the mean of the two duplicates.

4.3 Results

4.3.1 Incidence of heterozygosity at the loci used for clonality assays

Heterozygosity was determined using neutrophil DNA from 100 females at each of the polymorphic loci used to study clonality by XCIP analysis, 90% were heterozygous for the HUMARA, 37% for PGK, 36% for IDS, 34% for G6PD, 44% for FHL-1, 38% for p55 and 28% for the BTK assay. Overall, 90% were informative for at least one of the RNA assays (Figure 4.3), and of these 60% (54 out of 90) were heterozygous at more than one locus. Ninety seven percent were heterozygous for at least one DNA locus, with 30 out of 97 (31%) heterozygous for both PGK and HUMARA.

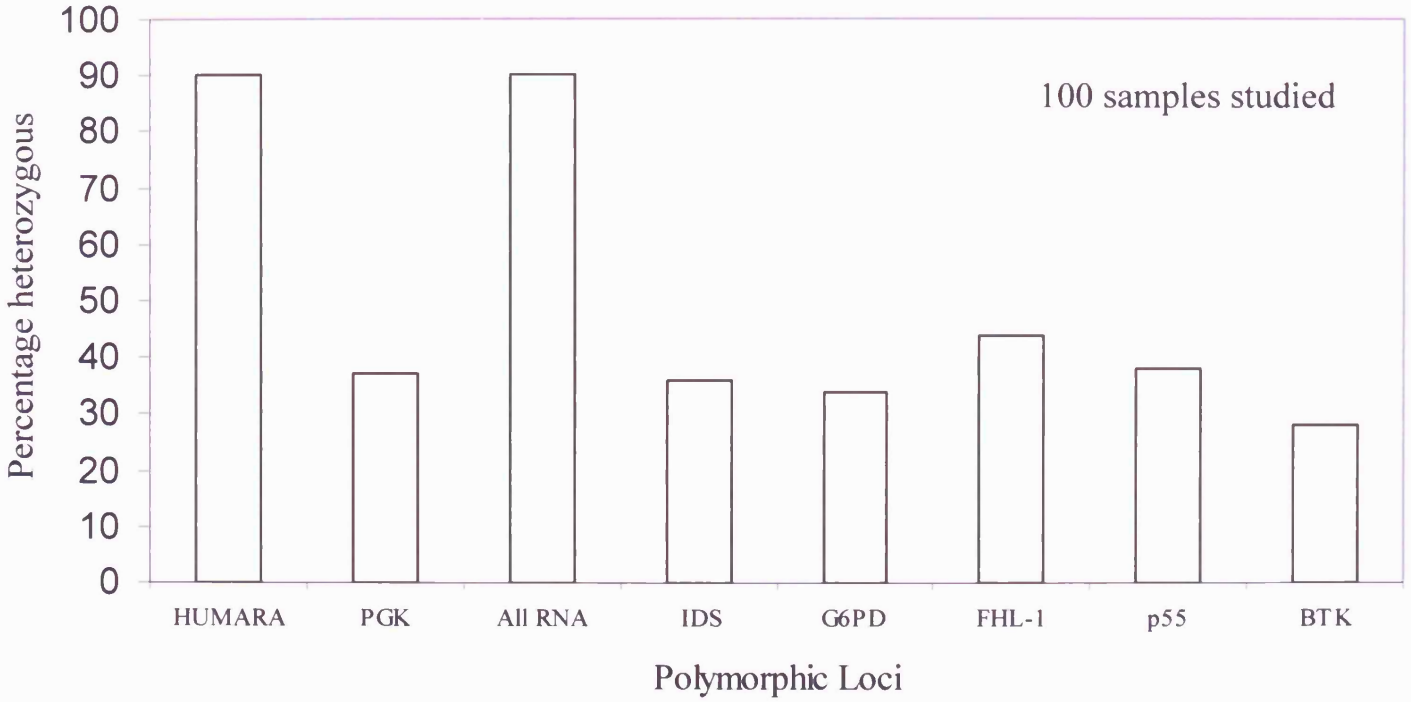

Figure 4.3
Percentage of samples heterozygous at each of the loci used for the clonality assays.

4.3.2 HUMARA results

Of the 92 female patients with persistent thrombocytosis, 80 had a diagnosis of ET according to the PVSG criteria. Of these patients 20 were shown to have clonal myelopoiesis by XCIP assay, 28 had polyclonal myelopoiesis and 29 were uninterpretable due to a constitutively imbalanced XCIP or because they had a clonal pattern and were more than 65 years of age. The remaining 3 patients were not informative for XCIP analysis. Therefore, 42% of interpretable patients had clonal myelopoiesis and 58% had polyclonal myelopoiesis. A representative HUMARA of one clonal and one polyclonal patient is shown in Figure 4.4.

Follow-up samples were obtained from 8 patients with clonal myelopoiesis at first test and 14 patients with polyclonal myelopoiesis at first test. Median age at first test for the clonal cohort was 55 years (range 10 – 61). Between one and 4 additional samples were obtained for each patient, median time since first test was 52 months (range 5-97) and mean time since first test 44 months. Diagnosis dates were available from 6 out of the 8 patients clonal at first test. Both median and mean time since diagnosis was 102 months (range 23-252). The polyclonal cohort had a median age at first test of 49 years (range 25 – 84). Between one and 5 additional samples were obtained for each patient, median time since first test was 54 months (range 10-102), mean time since first test 56 months. Diagnosis dates were available from 11 of the 14 polyclonal patients. Mean time since diagnosis was 99 months and median time since diagnosis was 78 months (range 45-188).

Clonality status did not change during follow up in any of the patients studied (Tables 4.4 and 4.5). The XCIPs obtained from neutrophils from all 8 patients clonal at first test remained imbalanced at >75% expression of one allele, T-cell results were balanced at <75% expression of one allele and the difference between T-cell and neutrophil results remained >20%. Median change in neutrophil result was 1.5% (range 0 – 16) (Figure 4.5).

Clonality status remained constant throughout the course of follow up for patients polyclonal at first test, and the median change of neutrophil XCIPs was 6% (range 0 – 25) for the polyclonal patient cohort (Figure 4.6). However, XCIPs from 4 patients did become imbalanced in neutrophil samples during the course of follow up. Patient 9 had a neutrophil XCIP result of 74%:26% expression of each allele at first test which changed by 6% to 80%:20% 48 months later and remained at 80%:20% after 87 months. At the time of first test patient 9 was 66 years of age and at last test was 73 years of age, therefore the change in neutrophil result could be due to age-related skewing (Gale *et al*, 1997). However, it is

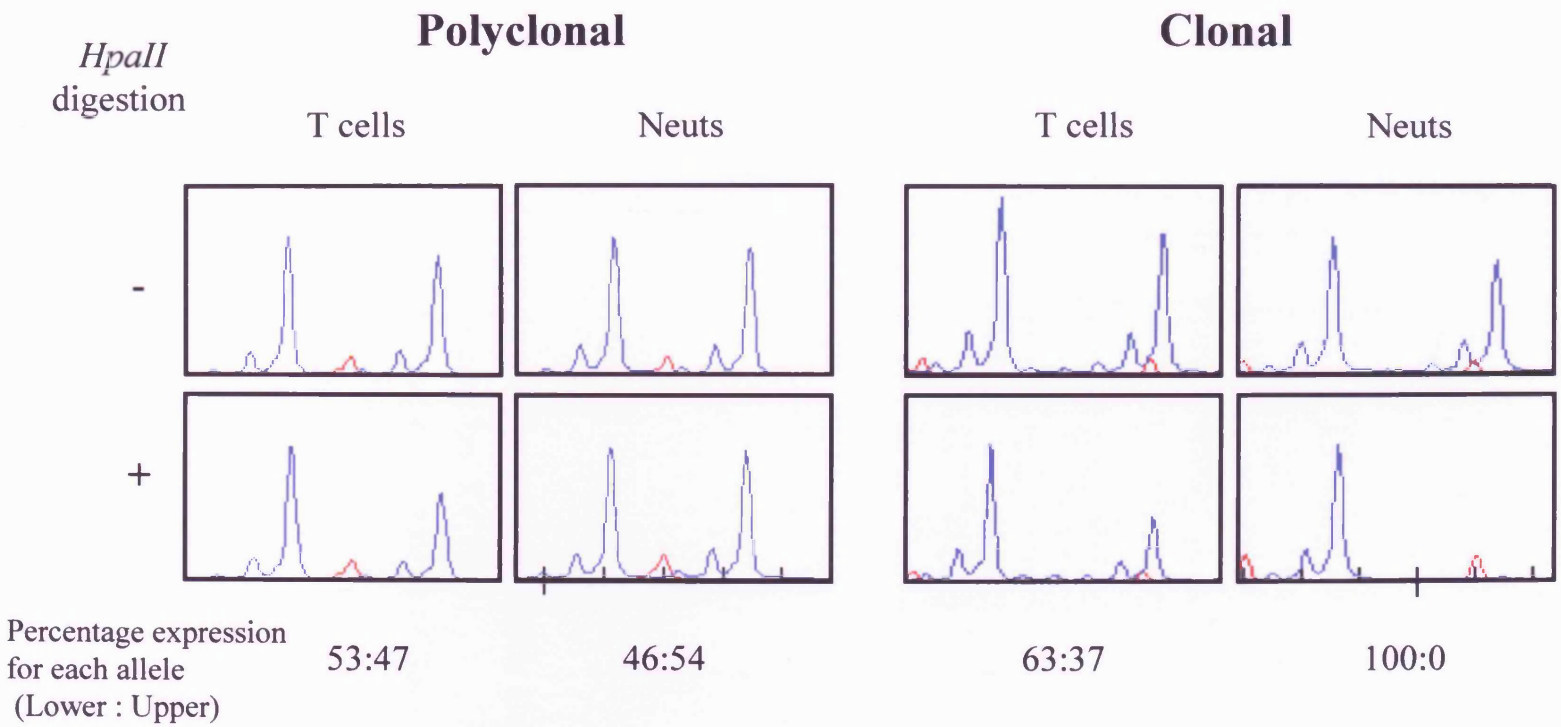

Figure 4.4
Representative XCIP from one clonal and one polyclonal ET patient using the HUMARA clonality assay
The blue trace represents PCR products and the red trace represents the size standard.

Patient Number	Cell Type	XCIP result at first test	XCIP results for subsequent tests *			
			1	2	3	4
1	T	27	31 (61m)	25 (74m)	27(85m)	25 (97m)
	N	0	0	4	3	6
2	T	52	57 (53m)	50 (55m)		
	N	1	100	100		
3	T	1	59 (54m)			
	N	91	89			
4	T	56	54 (50m)			
	N	11	26			
5	T	66	55 (17m)	58 (23m)		
	N	21	22	22		
6	T	62	71 (24m)			
	N	100	84			
7	T	40	45 (16m)			
	N	0	0			
8	T	71	68 (5m)	68 (19m)		
	N	100	100	95		

Table 4.4
HUMARA results for ET patients with clonal myelopoiesis at first test by XCIP

All results given as percentage expression of the smaller allele

T = T-cells N = Neutrophils

*Time from first test (months)

Patient Number	Cell Type	XCIP result at first test	XCIP results for subsequent tests *				
			1	2	3	4	5
9	T	64	35 (48m)	NA (74m)	NA (87m)		
	N	74	80	80	80		
10	T	45	53 (43m)	51 (67m)	NA (84m)	NA (102m)	
	N	51	64	67	66	68	
11	T	70	70 (47m)				
	N	51	76				
12	T	47	48 (56m)	NA (86m)			
	N	47	53	53			
13	T	38	40 (39m)	40 (60m)	NA (72m)		
	N	26	37	37	38		
14	T	31	14 (38m)				
	N	46	48				
15	T	67	65 (23m)	69 (35m)	63 (55m)	64 (71m)	58 (72m)
	N	64	59	62	61	60	60
16	T	65	61 (27m)				
	N	73	74				
17	T	58	60 (25m)				
	N	37	43				
18	T	17	35 (20m)	36 (22m)	38 (37m)	37 (50m)	
	N	36	19	21	31	23	
19	T	39	38 (52m)	38 (69m)	NA (85m)	41 (102m)	
	N	38	30	27	32	33	
20	T	41	42 (10m)				
	N	25	25				
21	T	25	NA (34m)				
	N	19	15				
22	T	30	36 (87m)				
	N	26	27				

Table 4.5
HUMARA results for ET patients with polyclonal myelopoiesis at first test by XCIP

All results given as percentage expression of the smaller allele

T = T-cells N = Neutrophils NA = Sample not available

*Time from first test in months

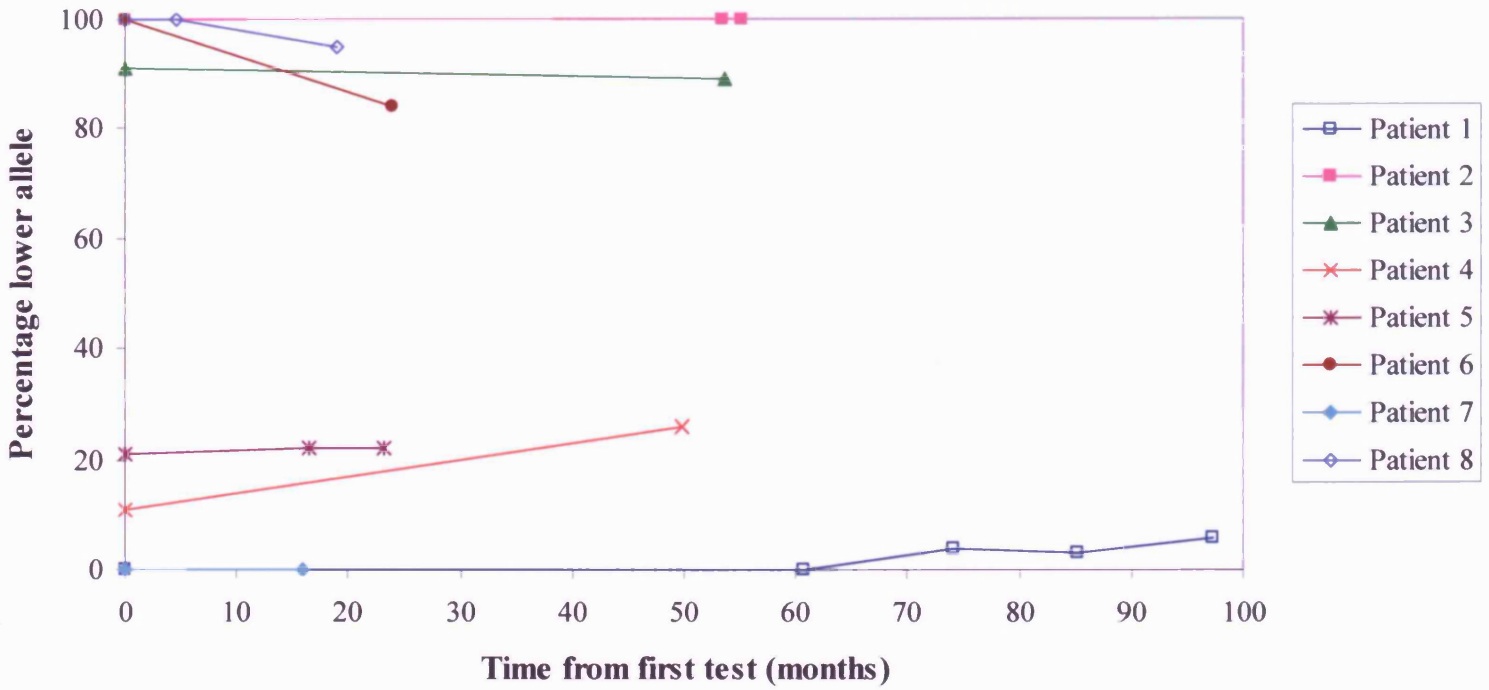

Figure 4.5
Graph of XCIP results from neutrophil samples obtained from ET patients clonal at first test.
All data was obtained using HUMARA

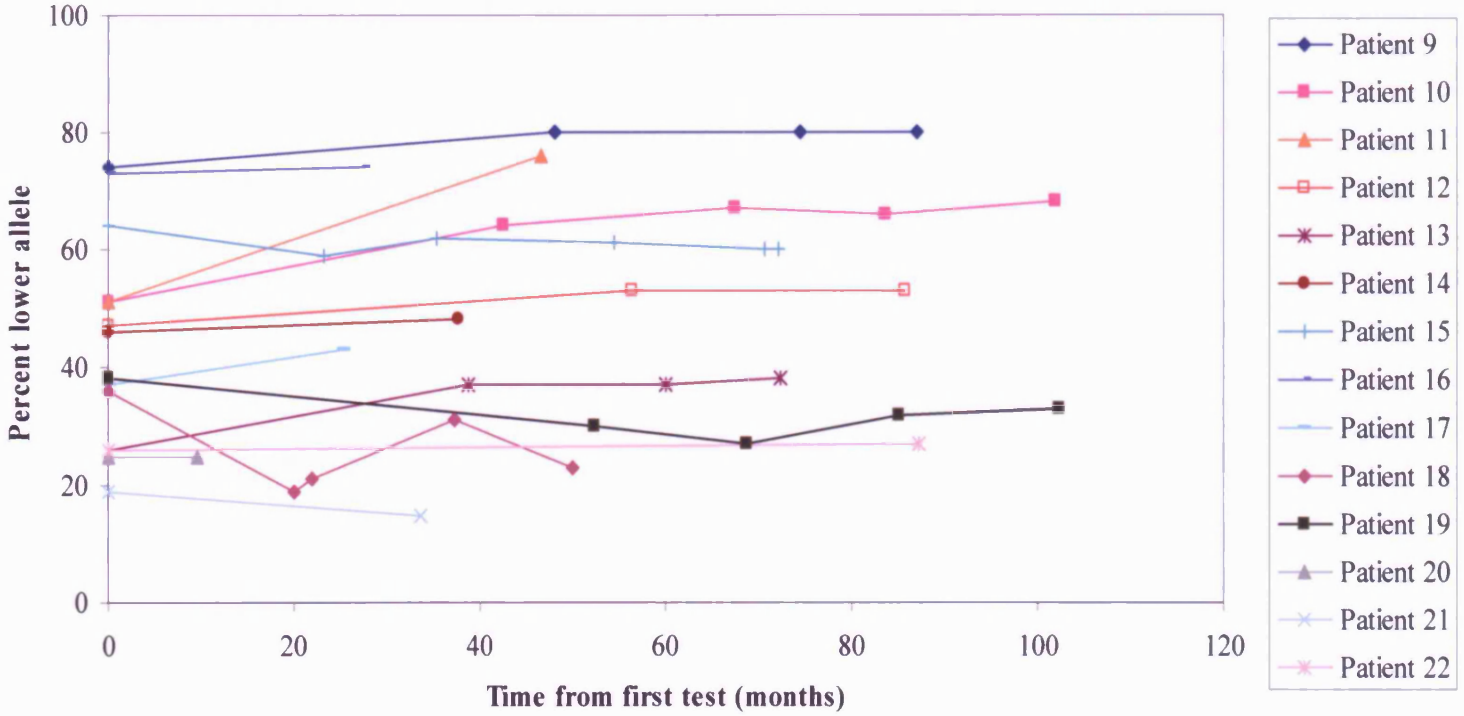

Figure 4.6
Graph of XCIP results from neutrophil samples obtained from ET patients polyclonal at first test.
All data was obtained using HUMARA

more likely that this change represents technical variation. The T-cell results for patient 9 were <20% different from neutrophils throughout the study, confirming that this patient was polyclonal.

The neutrophil XCIP results from patient 11 changed from 51%:49% expression of each allele at first test to 76%:24% 47 months later. However, this change was thought to be due to age-related skewing as at the time of last test patient 11 was 77 years of age. Also, the T-cell result was 70%:30% expression of each allele in both samples so the clonality status remained polyclonal in spite of the 25% change in neutrophil result.

The neutrophil XCIP results of patient 18 were less than 25% expression of the lower allele in 3 out of 5 samples studied. Neutrophil results of 36%:64%, 19%:81%, 21%:79%, 31%:69% and 23%:77% expression of each allele were obtained over the course of 50 months. The largest change in neutrophil result was 17%, and at no time point was the T-cell result >20% different from the neutrophil result. Therefore, clonality results from patient 10 were polyclonal throughout the course of follow up. It is possible that a handling error may have occurred with this sample. The T-cell result at first test was 17%:83% and the neutrophil result 36%:67%. Twenty two months later the T-cell result was 35%:65% and the neutrophil result was 19%:81%. All other T-cell results were consistent with the second sample, suggesting that the original T-cell and neutrophil samples may have been mislabelled.

The neutrophil XCIP results of patient 21 were less than 25% expression lower allele at both the original test (19%:81%) and 34 months later (15%:85%). However, the T-cell result for patient 21 at first test was 25%:75%. Therefore, patient 21 had a polyclonal myelopoiesis at both first test and after 34 months follow up as the T-cell XCIP was balanced and the neutrophil XCIP was less than 20% different from T-cell results.

All other patients with polyclonal myelopoiesis at first test had a balanced XCIP in both T-cells and neutrophils throughout the course of the study.

4.3.3 Comparison of RNA and DNA data

RNA clonality data was obtained from 10 patients with follow-up samples. Of the 10 patients, 4 were clonal and 6 were polyclonal at first test (Table 4.6). Five of the 10 patients were analysed at more than one time point resulting in a total of 18 samples being analysed by both DNA and RNA. Four of the 18 samples were analysed at more than one RNA locus. Therefore, a total of 24 samples were analysed in T-cells and neutrophils by

(a) Polyclonal

Patient Number	Cell Type	DNA 1	RNA 1a	RNA1b	RNA1c	DNA 2	RNA 2a	RNA2b	DNA 3	RNA 3
11	T	70	58	66		70	57	54		
	N	51	57	55		76	70	77		
	P		61	48			59	36		
12	T	48	49	58	58					
	N	53	49	56	56					
	P		53	65	50					
14	T	14	14							
	N	48	14							
	P		0							
15	T	69	62	62	58					
	N	62	68	61	69					
	P		58	41	55					
16	T	65	87			61	66			
	N	73	93			74	75			
	P		96				86			
18	T	17	19			35	25		36	24
	N	36	22			19	14		21	12
	P						3			9

(b) Clonal

Patient Number	Cell Type	DNA 1	RNA 1	DNA 2	RNA 2	DNA 3	RNA 3
1	T	27	2	31	31	25	28
	N	0	3	0	0	4	0
	P		6		0		
3	T	59	98				
	N	89	88				
	P		93				
5	T	66	15	55	20	58	19
	N	21	21	22	20	22	27
	P		5		1		4
7	T	45	4				
	N	0	0				
	P		4				

Table 4.6

Comparison of XCIP results using DNA and RNA clonality assays from patients with (a) polyclonal myelopoiesis and (b) clonal myelopoiesis

All results are given as percentage expression of the smaller allele

T = T-cells N = Neutrophils P = platelets

Samples collected at different times from the same patients are numbered 1, 2 or 3

Samples from one patient assesed by one or more RNA assays are indicated by a, b or c

The RNA assay used to asses clonality is colour coded:

Green = p55 Red = IDS Blue = BTK Purple = G6PD

both HUMARA and RNA analysis, giving a total number of DNA:RNA comparisons of 48. Overall, DNA and RNA results were similar, with correlation coefficients for XCIP results of r= 0.87 and r^2= 0.76. The median difference between DNA and RNA results was 6.5% (range 0-34), mean difference was 8.3% and modal difference 1%.

Sixteen T-cell and 16 neutrophil results were obtained from DNA and RNA from the 6 polyclonal patients. Only 2 of the 32 DNA verses RNA comparisons were greater than 20% different. Neutrophils from patient 14 had a balanced XCIP of 48%:52% expression of each allele by DNA analysis, but by RNA analysis the XCIP was imbalanced at 14%:86% (BTK assay), a change of 34%. The T-cell sample also had an XCIP of 14%:86% so the patient was considered to be uninterpretable by RNA analysis due to a constitutively imbalanced XCIP. T-cell and neutrophil XCIP results from the first sample from patient 16 assayed by HUMARA were 65%:35% and 73%:27% expression of each allele respectively, but by RNA (p55) analysis were 87%:13% and 93%:7% respectively. This would alter the clonality status of this patient from polyclonal by DNA analysis to uninterpretable by RNA analysis. However, the RNA results from the follow up sample from patient 16 were less than 5% different from DNA results for both T-cell and neutrophils (Table 4.6a), suggesting that the original RNA result may have been incorrect, although the reason for this is unknown.

Four ET patients with clonal myelopoiesis by DNA analysis were investigated by RNA analysis; 8 T-cell and 8 neutrophil results were obtained (Table 4.6b). The RNA neutrophil results were less than 5% different from DNA neutrophil results in all samples tested. However, 6 of the 8 T-cell samples tested had RNA results that were more than 20% different to DNA results. For patient 1, 3 RNA results were obtained using the IDS assay at 3 different time points. All RNA results were equivalent to DNA results except the T-cell result from the initial sample, where the XCIP was 27%:73% expression of each allele by DNA assay, but 2%:98% by RNA. This was probably due to platelet contamination of the T-cell fraction as cytospins of T-cells from the first sample showed large numbers of platelets adhered to the T-cells. As the XCIP obtained from the platelet sample was 6%:94%, contaminating RNA from platelets could account for the discrepancy between DNA and RNA results in patient 1.

Patients 3 and 7 were informative for BTK. DNA and RNA neutrophil XCIP results were comparable, but T-cells results were not: XCIPs from T-cells from patient 3 were 59%:41% expression of each allele by DNA and 98%:2% by RNA, XCIPs from T-cells

from patient 7 were 45%:55% by DNA and 4%:96% by RNA. However, as the T-cell XCIP results from RNA were similar to neutrophil and platelet results from both RNA and DNA, this suggests that contamination of the purified T-cell fraction by platelets may be the cause of the aberrant T-cell results.

Patient 5 was informative for the p55 RNA clonality assay. Results were obtained from three samples for patient 5 and in each case the neutrophil results from DNA and RNA were similar. However, T-cell XCIP results obtained from the p55 assay were imbalanced and did not match the balanced XCIP results obtained from T-cells by HUMARA. T-cell XCIP results from p55 were 15%:85%, 20%:80%, and 19%:81% expression of each allele, and T-cell XCIP results from HUMARA were 66%:34%, 55%:45% and 58%:42% expression of each allele. The T-cell results did, however, closely match results from the myeloid lineage, the neutrophil XCIP results by RNA assay were 21%:79%, 20%:80% and 27%:73% expression of each allele and by HUMARA were 21%:79%, 22%:78% and 22%:78%. Again this suggests contamination of the T-cell fraction with cells of the myeloid lineage.

In this small cohort of ET patients clonal by DNA analysis, 6 out of the 8 RNA XCIP results were uninterpretable due to imbalanced T-cell XCIPs. This was thought to be due to contamination of the polyclonal T-cell fraction by clonal platelets.

4.3.4 HUMARA in male ET patients

To test directly whether hypermethylation at the human androgen receptor locus affected HUMARA results, male ET patients were assayed. As males only posses one X-chromosome, it is always active and therefore should be unmethylated. This allele should be completely digested by *Hpa*II leading to the absence of any sample peaks in the *Hpa*II digested aliquot. Hypermethylation of the CpG island located in the recognition sequence of *Hpa*II in the HUMARA PCR product would lead to a peak in the *Hpa*II positive fraction. HUMARA was carried out on DNA obtained from the neutrophils of 11 male ET patients. No measurable product peak was present in the *Hpa*II positive aliquot in 10 (89%) samples (Figure 4.7A). In one sample a peak was observed in the *Hpa*II positive aliquot. The peak was very small compared to the aliquot with no *Hpa*II, representing approximately 4% of the height of the peak in the *Hpa*II negative fraction (Figure 4.7B). If hypermethylation does occur in the human androgen receptor in ET patients, it is at a very low level which would have little or no effect on clonality results.

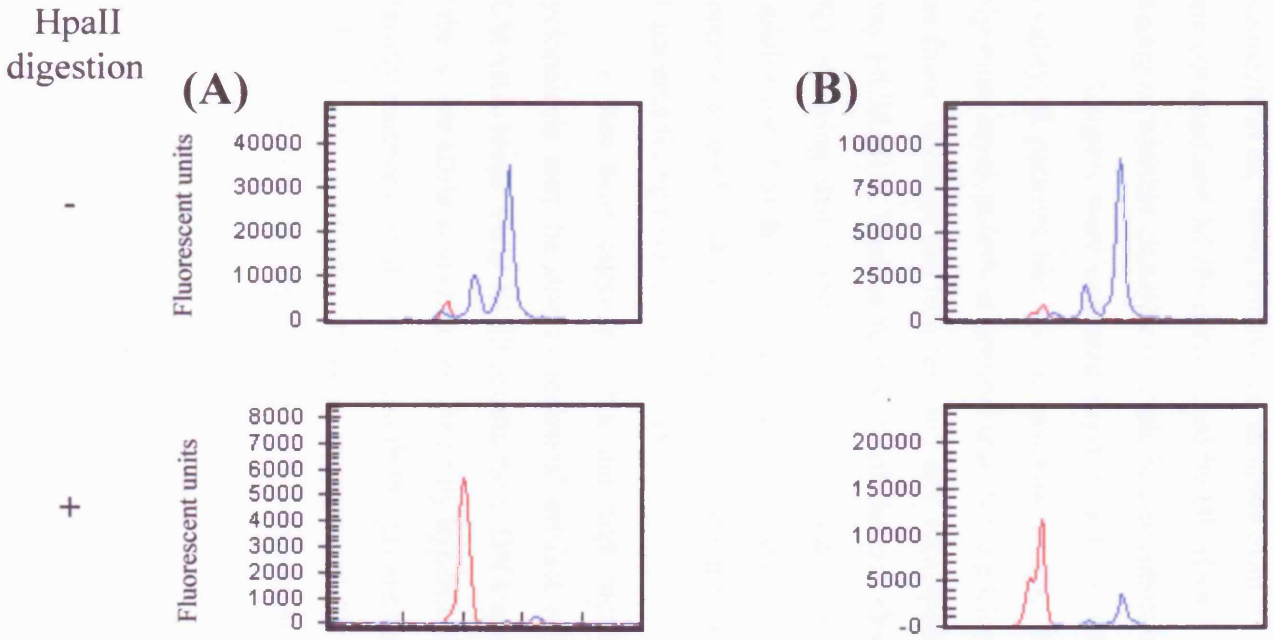

Figure 4.7
Electrophoretograms of HUMARA PCR carried out on neutrophil DNA from male ET patients, with or without pre-incubation with *Hpa*II
(A) – Representative electrophoretograms of the 10 male ET patient samples in which no PCR product was obtained from the *HpaII* digested aliquot.
(B) – Electrophoretograms of the male ET patient sample in which PCR product was obtained in the *HpaII* digested aliquot.
The blue trace represents PCR product, and the red trace represents the size standard.

4.4 Discussion

Use of XCIPs to indirectly assess clonality in ET patients has shown that approximately 40% of patients have polyclonal myelopoiesis. Those patients with polyclonal myelopoiesis appear to be at a lower risk of thrombotic events than ET patients with clonal myelopoiesis (Harrison *et al*, 1999a; Chiusolo *et al*, 2001; Shih *et al*, 2002; Vannucchi *et al*, 2004). Follow-up samples from ET patients previously tested for clonality were obtained and XCIPs determined by HUMARA (and RNA assay where informative) to investigate whether clonality is stable in this cohort of patients.

Samples were collected from 22 ET patients who had previously been tested for clonality, 8 patients had clonal myelopoiesis at their original test and 14 patients had polyclonal myelopoiesis at original test. No significant change in T-cell or neutrophil XCIP was found between the first test and any subsequent test, using the DNA-based clonality assay HUMARA. Median follow up of the polyclonal cohort was 61 months (range 10 – 102), showing that clonality status was stable in these patients. Diagnosis dates were available for 11 of the polyclonal patients, the latest samples for this cohort were median 78 months (range 45 – 188) post diagnosis, indicating that clonality status can remain stable in ET patients for up to at least 16 years.

It has been suggested that the high incidence of ET patients with polyclonal myelopoiesis may be due to technical artifact related to hypermethylated DNA at the HUMARA locus. Only XCIP results from DNA assays would be affected, since digestion of the active allele would be prevented by hypermethylation (Figure 4.1). This may lead to a falsely balanced XCIP, even though the patient may have an underlying clonal disorder. As RNA assays directly measure the amount of each allele that is transcribed, while expression levels may be reduced with hyper-methylation, relative expression of the two alleles should remain unchanged.

All ET patients in the cohort polyclonal by DNA assay were also polyclonal by RNA assay. This suggests that hypermethylation does not affect XCIPs obtained from DNA clonality assays. In clonal patients, RNA results from neutrophils were similar to DNA results. However, 6 out of 8 T-cells results which were balanced by DNA analysis were imbalanced by RNA analysis. Many patients with ET have activated platelets which are more sticky than non-activated platelets. This can lead to the formation of platelet

clumps and cause platelets to adhere to leukocytes. These platelet/leukocyte interactions can lead to contamination of purified T-cell and neutrophil fractions by large numbers of platelets (Griesshammer *et al*, 1999; Villmow *et al*, 2002; Falanga *et al*, 2000). This does not affect the DNA assay as platelets are anucleate, or the neutrophil fraction of RNA assays as platelet and neutrophil fractions are both of the myeloid lineage and would therefore be expected to have the same XCIP. However, the T-cell fraction of clonal patients has a different XCIP to platelets, so platelets in the purified T-cell fraction may lead to an imbalanced XCIP in T-cells from clonal patients.

Expression levels of a specific gene may vary between cell types. Therefore, low level contamination of a purified cell lineage by cells from a second lineage can have a large effect on clonality results. One study has shown that the number of IDS gene transcripts from one T-cell were equivalent to those from 6 neutrophils or 800 platelets (Harrison *et al*, 1998a). For the BTK gene, expression in T-cells is less than 1% of that seen in B-cells (Smith *et al*, 1994). As B-cells in clonal ET patients are also clonal (Raskind *et al*, 1985), a small level of B-cell contamination in purified T-cell samples may account for the imbalance in XCIP obtained by BTK. RNA clonality assays should therefore be interpreted with caution, as incorrect results can occur due to contamination from other cell lineages.

To further analyse hypermethylation in ET patients at the HUMARA locus, male ET patients was investigated for methylation status at the human androgen receptor gene. As males only have one X-chromosome, which will be active, incubation with *Hpa*II should digest all X-chromosomal DNA between the primer annealing sites, preventing PCR product formation. Any PCR product formation would be suggestive of hypermethylation. After *Hpa*II digestion and PCR of neutrophil DNA from the 11 male ET patients, only one had sufficient PCR product to be detected. In this patient the PCR product represented approximately 4% of the amount of product measured without *Hpa*II digestion. It is possible that this is a result of incomplete digestion by *Hpa*II in this specific sample, but if this is not the case then this suggests that any hypermethylation at the HUMARA locus in ET is rare and of a low level and is unlikely to be sufficient to alter clonality results. Therefore, HUMARA data is usually similar to RNA data, especially in polyclonal patients, and where differences occur this is often due to technical problems, frequently caused by sample contamination by different cell lineages.

In summary, these data demonstrate that the high proportion of ET patients reported with polyclonal myelopoiesis is not due to technical artifact and HUMARA accurately reflects the biological situation. Clonality status in polyclonal and clonal ET patients appears stable during long term follow up by XCIP. This suggests that ET can be divided into at least 2 subgroups: a group with clonal myelopoiesis - at higher risk of developing thrombotic complications, and a group with polyclonal myelopoiesis, which remains polyclonal throughout the clinical course of the disease and is at a lower risk of thrombotic complications than clonal patients. It may be an advantage to investigate clonality status in all female ET patients as clonality status, considered in combination with other indicators of risk, may influence a treatment regimen. In fact, in a retrospective study of 27 ET patients, one centre has shown that the 8 polyclonal ET patients received less cytoreductive treatment than the 19 clonal patients, supporting the view that clonality testing should be used more widely (Briere *et al*, 1999).

The molecular basis of polyclonal ET may be due to stimulation of the MK lineage by proliferation and/or survival factors from external sources, such as an increase in TPO expression (see Chapter 5). However, clonal ET would be expected to arise by one or more acquired alterations in a stem cell or early myeloid progenitor cell. Therefore it would be helpful for studies investigating the molecular basis of ET to asses clonality status in their patient cohort to enable investigators to study ET both as one single disease and as two distinct clinical entities based on clonality status.

Chapter 5

Mutation screen of the 5' UTR of the thrombopoietin gene in ET patients

5.1 Introduction

5.1.1 Historical perspective

The name TPO was first coined in 1958 to describe the humoral regulator of platelet production. However, 36 years passed before the TPO protein was isolated and the gene cloned. The isolation of TPO was made possible by the discovery of an acute leukaemogenic retrovirus (MPLV) which induced a myeloproliferative syndrome in mice (Wendling *et al*, 1986). The molecular cloning of MPLV showed it to be a truncated form of a new member of the haemopoietic growth factor receptor superfamily (Souyri *et al*, 1990). The murine and human forms of the receptor, termed Mpl, were subsequently cloned (Vigon *et al*, 1993) and were shown to contain characteristic features of members of the superfamily, i.e. organisation of the cytoplasmic domain into 200 amino acid cytokine receptor homology domains (CRH) containing four distinctive cysteine residues at their N-terminal end. Each CRH can be subdivided into two modules of 100 amino acids with the C-terminal domain containing 2 sets of 7 anti-parallel β strands flanking a Trp Ser X Trp Ser (WSXWS) box close to the transmembrane domain (Cosman, 1993; Kishimoto *et al*, 1994). The extracellular portion of Mpl consists of 2 CRHs.

Mpl was left as an orphan receptor, as the Mpl ligand was still to be identified. Subsequent experiments identified Mpl as important in promoting MK development. RT-PCR analysis showed that c-mpl transcripts (the gene coding Mpl protein is called c-mpl) were limited to groups of cells that contained MKs or their precursors, and to cell lines that could be induced to display MK characteristics (UT-7, MO7e, TF-1, HEL, DAMI and KU-812) (Methia *et al*, 1993). Chimeric proteins, formed by fusing the cytoplasmic portion of Mpl to the extracellular portion of IL-4 or G-CSF, demonstrated that the cytoplasmic portion of Mpl could transmit a proliferative signal in haematopoietic cells (Skoda *et al*, 1993; Vigon *et al*, 1993). Also, CFU-MK derived from human BM CD34+ cells were selectively inhibited by addition of c-mpl antisense oligonucleotides to CD34+ cells *in vitro* (54-81% inhibition) (Methia *et al*, 1993). The expression of Mpl decreased by 50-70%. However, erythroid, granulocytic and monocytic colony formation was unaffected, demonstrating the specificity of signalling via Mpl.

In 1994, four separate groups independently reported the isolation and cloning of TPO, also termed Mpl ligand or MK growth and development factor (MGDF), using three

different techniques. Two groups used Mpl to purify TPO (Bartley *et al*, 1994; de Sauvage *et al*, 1994). Affinity chromatography was used, based on immobilized recombinant Mpl, to purify TPO from porcine and canine plasma respectively. Another group identified TPO by constructing a Mpl ligand dependent cell line and screening a cDNA expression library (Lok *et al*, 1994). Finally, using a model of thrombocytopenia in sheep as a source of TPO-enriched plasma, conventional protein fractionation methods were used to purify a protein which was capable of promoting MK differentiation (Kuter *et al*, 1994).

5.1.2. Location, structure and expression patterns of the TPO gene

The TPO gene locus was mapped to the long arm of chromosome 3 at 3q26-27 by fluorescent *in situ* hybridisation (Chang *et al*, 1995; Foster *et al*, 1994; Gurney *et al*, 1995; Sohma *et al*, 1994). The TPO polypeptide contains 353 amino acids. The first 22 amino acids are a signal peptide and the remainder of the peptide can be divided into two domains: a 155 amino acid EPO-like domain, which shares 46% overall sequence similarity with EPO and which binds c-mpl (Bartley *et al*, 1994), and a 177 amino acid carboxy-terminal domain which is unique to TPO (Figure 5.1). The removal of the carboxy-terminal domain does not affect the activity of the protein but does reduce bioavailability (Hokom *et al*, 1995).

The TPO gene contains 2 non-coding exons and 5 coding exons. The gene promoter contains no TATA box and there are no other regulatory domains present (Chang *et al*, 1995). TPO expression is therefore thought to be constitutive. The 2 non-coding exons both contain promoter regions. The beginning of exon 1 contains a promoter (P1), which produces a full length TPO transcript. The second promoter (P2) lies within exon 2 and a shorter transcript is produced which lacks all of exon 1 and part of exon 2. The transcript produced from P2 is more efficiently translated than that produced from P1 due to the lack of transcription initiation sites (AUGs) from exon 1 (see below). The most common transcript found in liver is produced from P2, with only 10% of transcripts produced by P1 (Ghilardi *et al*, 1998) (Figure 5.1). Expression of TPO occurs primarily in the liver, but it is also produced in kidney and smooth muscle, as shown by northern blot and RT-PCR, and to a lesser extent in the spleen and bone marrow.

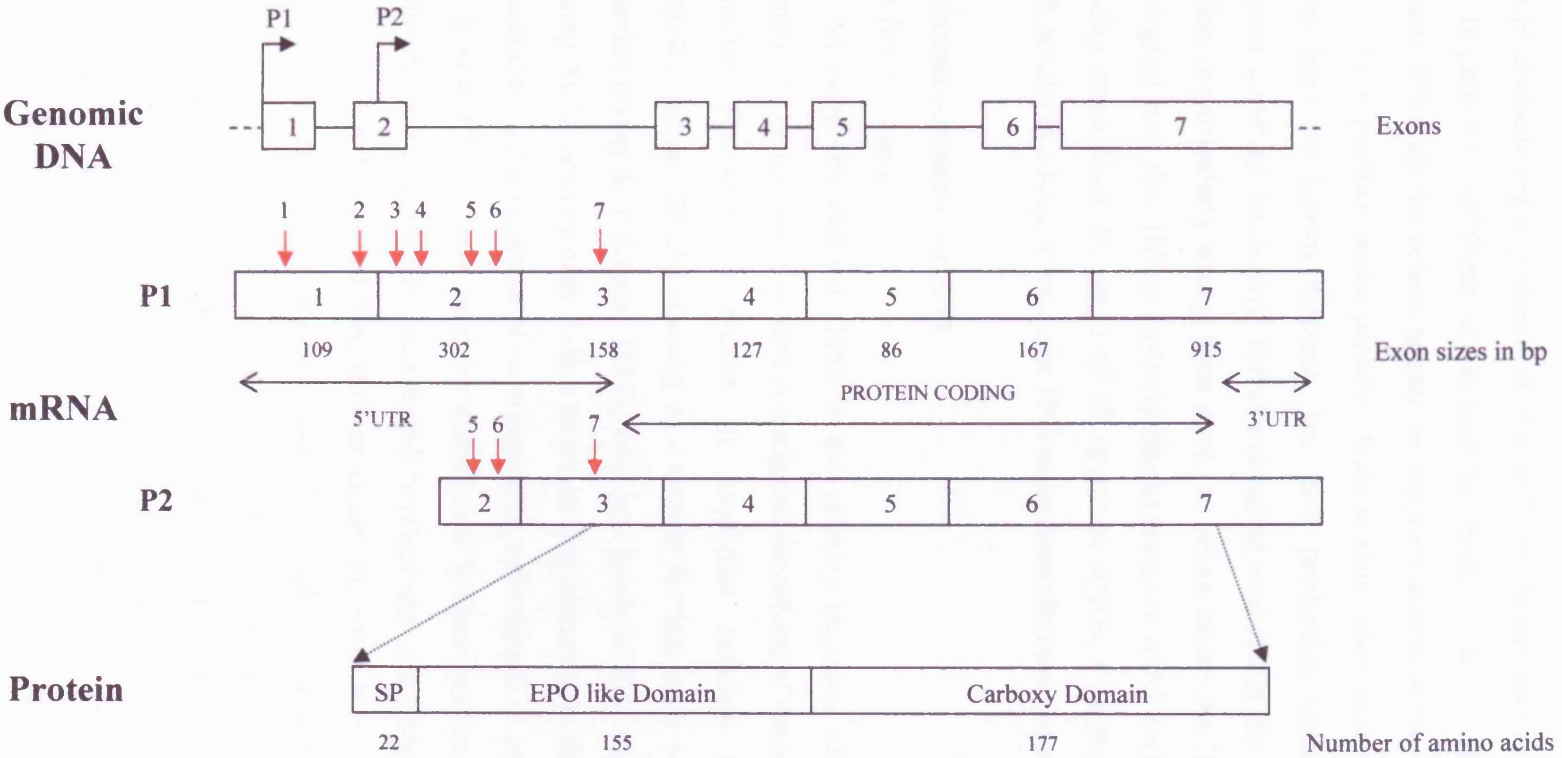

Figure 5.1
TPO gene organisation.
The organisation of TPO genomic DNA, the mRNA transcripts produced by each promoter, and protein structural motifs, showing the location of the two promoters on genomic DNA The 7 AUG start sites are indicated by vertical red arrows above the transcript produced by each promoter

As well as TPO mRNA being constitutively expressed, production of TPO protein is highly suppressed at the translational level. The 5' UTR of the full-length mRNA transcript contains eight potential AUGs and the shorter transcript from P2 contains 4 AUGs (Figure 5.1). Of these AUGs only the most 3' site in exon 3 (AUG 8) produces functional TPO, all the others appear to suppress access to the physiological start site. AUGs 1 to 7 produce small peptides from a short open reading frame (ORF). These peptides have no known function, but their production causes competition for the translation initiation machinery, thereby reducing availability for AUG 8 (Kozak, 1989). Inhibition is particularly strong from start site seven where the ORF extends beyond the physiological start site. This completely blocks access to AUG 8 while the ORF from AUG 7 is being transcribed. Removal of all upstream AUGs, thus removing all inhibition to AUG 8, results in a large increase in TPO expression (Wiestner *et al*, 1998).

5.1.3 Functional studies of TPO

5.1.3.1 In vitro studies

As originally defined, TPO is the primary regulator of MK proliferation and differentiation. A number of groups investigated the effects of the newly discovered protein on murine BM cells to ensure its properties matched this definition. Using acetylcholinesterase (AChE) staining as a marker for late stage MKs (the first identified MK marker (Long & Williams, 1981), very low levels of TPO (750pg/ml) were better at promoting AChE activity after 6 days of serum free culture than 5000pg/ml IL-6 or IL-11, either alone or in the presence of co-stimulatory molecules IL-3 or KL (Kaushansky *et al*, 1994). IL-6 and IL-11 were used for comparison in these studies as they had previously been shown to stimulate MK growth and development and were, themselves, candidate proteins for TPO. The use of 50ng/ml was needed for either IL-6 or IL-11 to approach the level of activity induced by the low dose TPO, demonstrating the potency of TPO on megakaryocytopoiesis. Individual AChE-stained cells were assessed and it was shown that both the number and the size of MKs were greater in the presence of TPO than IL-6 or IL-11. TPO induced a higher level of ploidy than IL-6 or IL-11 when measured by FACS analysis, with modal ploidy levels of 32 or 64N for TPO-induced cells, but only approximately 8-16N for IL-11-induced cells. TPO was also shown to be a potent colony-stimulating factor. Cell culture of murine BM cells in 10% horse serum demonstrated that recombinant TPO could support CFU-MK growth in a dose-dependent fashion, and the

ploidy level of 30-35% of those MKs was greater than or equal to 64N (Broudy *et al*, 1995). These studies demonstrated that TPO is a potent proliferation and maturation factor during megakaryocytopoiesis.

TPO also stimulates end stage maturation and platelet formation. Expression of platelet-specific membrane proteins can be stimulated by TPO, including GpIb and GpIIb/IIIa (Bartley *et al*, 1994; de Sauvage *et al*, 1994). Electron micrograph studies of murine BM cells have shown that TPO induced the formation of a mature peripheral zone in MKs which was devoid of organelles and a well-developed demarcation zone. TPO stimulated the fragmentation of the demarcation zone into platelets (Kaushansky, 1995).

5.1.3.2 In vivo studies

TPO can also stimulate MK proliferation and maturation *in vivo* as measured by platelet counts. Administration of 50ng TPO to wild type BALB/c mice led to greater than fourfold increase in platelet production (Lok *et al*, 1994). Also, in healthy human volunteers, a single subcutaneous injection of 3μg/kg pegylated recombinant human MGDF (PEG-rHuMGDF) led to an increase in platelet counts from normal levels of 237±41 $x10^3/\mu l$ to peak counts of 522±90 $x10^3/\mu l$ ($p<.0001$) by day 12. The platelet counts normalised at 28 days in response to a decrease in serum TPO levels during the course of the experiment (Harker *et al*, 2000).

Thrombocytopenia can be abrogated by the administration of TPO. In a murine model, made thrombocytopenic by lethal irradiation prior to BM transplant (BMT), administration of 50μg/kg/d PEG-rHuMGDF over 17 days caused accelerated platelet recovery. Treated mice recovered sooner than control mice injected with a PBS carrier solution containing 0.1% BSA, with platelet counts reaching $300x10^3/\mu l$ by day 9 and over $1000x10^3/\mu l$ by day 12 compared to day 11 and day 16 respectively in control mice (Molineux *et al*, 1996). The severity of platelet nadir was also decreased with TPO treatment. Mice treated with 30μg/kg/d of PEG-rHuMGDF had a platelet nadir at 8% of pre-treatment levels compared to 4% in untreated animals (Kabaya *et al*, 1996).

In patients with thrombocytopenia, TPO administration can also lead to an increase in platelet counts. A phase one study of rhTPO administered after autologous BMT showed an average time to platelet recovery of 18 days, this was significantly quicker than historical studies where platelet recovery took 40 days on average (Wolff *et al*, 2001).

Breast cancer patients undergoing autologous BMT have also been studied in randomised, double blind trials of PEG-rHuMGDF (Schuster *et al*, 2002). Seventy five patients were randomised to receive placebo or PEG-rHuMGDF. Platelet recovery to $20x10^9/l$, and platelet transfusion requirements were significantly reduced in patients receiving PEG-rHuMGDF (18-19 days) compared to placebo (22 days).

Thus TPO stimulates the proliferation and maturation of MKs and the production of platelets both *in vitro* and *in vivo*, demonstrating that TPO is the primary regulator of megakaryocyte proliferation and differentiation.

5.1.4 Regulation of TPO levels in the circulation

As TPO expression is thought to be constitutive, there must be other regulatory mechanisms which control its circulating levels. However, these control mechanisms are not yet fully understood. The binding of TPO to Mpl on the surface of MKs and platelets removes TPO from the circulation (Li *et al*, 1999), thus an increase in MK and platelet mass leads to an increase in available Mpl and circulating TPO levels decrease. This is known as the sponge theory, and forms a negative feedback mechanism controlling circulating TPO levels in response to alterations in platelet numbers. It was first demonstrated by measurement of TPO levels in plasma from rabbits treated with busulphan to induce thrombocytopenia (Kuter & Rosenberg, 1995). TPO plasma levels increased after busulphan treatment as the platelet mass declined. The plasma levels of TPO increased inversely and proportionally to platelet levels, peaked as the platelet counts reached nadir, and decreased as platelet counts returned to normal. Transfusion of platelets into thrombocytopenic rabbits around the platelet nadir also caused the elevated TPO levels to decrease, suggesting that TPO was removed from the circulation by the increased platelet mass acting like a sponge. This effect has also been demonstrated in patients during chemotherapy, where serum TPO levels changed reciprocally with platelet counts. Transfusion of platelets around the time of platelet nadir caused an immediate drop in serum TPO levels as the platelet count increased (Shinjo *et al*, 1998). This effect was only temporary, and as the platelet counts reduced the TPO levels rose. Platelets obtained from these patients post-transplant, i.e. in a thrombocytopenic state, showed lower binding of biotinylated TPO than donor platelets prior to transfusion. This indicated that the TPO receptors were saturated by the excess endogenous TPO in patients with high serum levels.

A number of factors may also be involved in the control of TPO regulation which are unrelated to the sponge theory.

IL-6 stimulates the differentiation of MKs *in vitro* and *in vivo* which leads to an increase in platelet counts (Chapter 6.1.3). However, IL-6 may augment platelet production via its interaction with TPO. In the hepatoma cell line HepG2, addition of 1pg/ml to 1ng/ml IL-6 resulted in an increase of TPO mRNA expression in a dose-dependent manner. Mice treated with IL-6 developed thrombocytosis, with platelet counts at their peak 2.5 fold higher than untreated mice, and this was accompanied by an increase in hepatic TPO mRNA expression. Neutralization of TPO by rabbit anti-TPO polyclonal antibody prior to IL-6 treatment, abrogated the thrombocytosis. Also, cancer patients treated with 30mg/kg/d IL-6 for 5 days showed an increase in plasma TPO levels, from undetectable before treatment to 630±110pg/ml on day 6 (Kaser *et al*, 2001). These studies suggest that circulating TPO levels may be influenced by IL-6.

As well as IL-6, proteins contained in the α-granules have been shown to affect TPO expression. Platelet derived growth factor (PDGF) and fibroblast growth factor-2 (FGF-2) increased the expression of TPO mRNA in cultures of normal BM stromal cells by 2 and 1.5 fold respectively in a dose dependent manner. However, the opposite was shown for platelet factor 4 (PF4), TSP and TGFβ1. One to 2 μg/ml PF4 and TSP significantly inhibited TPO mRNA and protein expression compared to controls, as did 50 to 100ng/ml TGFβ1 (Sungaran *et al*, 2000). Whole platelet lysates suppressed TPO expression, suggesting that the negative effect of the complete contents of the platelet α-granules is greater that the positive, and that platelets may play a negative role in their own proliferation and maturation by modulating TPO expression.

Contrary to the evidence described above using high concentrations of TGFβ1, lower concentrations may lead to up-regulation of TPO mRNA in BM stromal cells. TGFβ1 is a potent inhibitor of megakaryocytopoiesis and is discussed at length in chapter 6 (6.1.1). Addition of 0.1-2ng/ml TGFβ1 to cultured BM stromal cells from normal individuals led to a dose-dependent increase in TPO mRNA up to 7 times normal levels as measured by quantitative RT-PCR. Media from cultures containing TGFβ1-treated cells contained more TPO (37.4±4.6pg/ml and 162.7±25.4pg/ml at 24 and 48 hours respectively) than untreated cells (18.3±2.4 and 30.4±3.7pg/ml), showing that the increase in TPO mRNA led to a higher level of TPO protein expression. This effect could be blocked by

addition of a TGFβ blocking antibody prior to treatment, showing that the effect was due to TGFβ1 alone (Sakamaki *et al*, 1999). This evidence suggests that TGFβ1 can up-regulate TPO expression in BM stromal cells. As TGFβ1 is a potent inhibitor of megakaryocytopoiesis, a negative feedback loop has been suggested whereby TGFβ1 stimulates TPO expression in BM stromal cells. This in turn stimulates the expression of TGFβ receptors (TGFβR) I and II on the surface of maturing BM MKs. Bioavailability of TGFβRI and II is increased facilitating TGFβ1 binding to MKs which leads to inhibition of megakaryocytopoiesis.

Clearly a number of mechanisms may be involved in TPO regulation. The sponge theory accounts for most of the changes in TPO circulating levels, but other regulatory mechanisms may be important in controlling TPO expression and ultimately platelet number, particularly in the BM micro-environment.

5.1.5 TPO levels in Thrombocytosis

The sponge theory accounts for the changes in serum/plasma TPO levels in the normal and thrombocytopenic state. However, in patients with thrombocytosis this is not always seen. Several diseases characterised by a thrombocytosis show a normal or elevated circulating TPO level.

Kawasaki disease (KD) is an acute febrile vasculitic syndrome seen in children and is accompanied by a thrombocytosis. TPO was detected in 26 out of 31 patients (mean 173pg/ml, range 89-249pg/ml) using an ELISA-based technique that was not sensitive enough to pick up TPO in haematologically normal individuals, thus TPO was elevated in the majority of KD patients with thrombocytosis (Miura *et al*, 1998). Patients with the cMPD agnogenic myeloid metaplasia (AMM), a disease characterised by BM MK hyperplasia and atypical MK clusters, have also been reported to have elevated TPO levels of 693.2±82.66pg/ml compared to normal levels of 185.80±26.62pg/ml ($p<0.0001$) (Wang *et al*, 1997).

A number of major studies have reported circulating TPO levels in patients with ET and their results are summarised in Table 5.1. All reports showed that circulating TPO levels in ET patients are equal to or higher than levels in normal individuals. Eight of the

Reference	Diagnosis	n=	Mean (± SD)	Median	Range	Source (units)
Harrison *et al* (1999)	N ET RT	12 18 8	122 ±69 162 ±138 140 ±137	110 118 69	39-242 <15-483 22-375	Plasma (pg/ml)
Cerruti *et al* (1997)	N cMPD RT	32 32 70		156.7 246.2 287	62.2-352.7 93.5-4596 82.7-1960	Serum (pg/ml)
Karakus *et al* (2002)	N cMPD RT	16 24 21		62.65 467 100.5		Plasma (pg/ml)
Pitcher *et al* (1997)	N ET RT&NEM	96 25 16	80.1 ±5 203.9 ±56.5 237.1 ±46.7	64.4 127.2 77.1	22.2-255.9 77.5-1551.9 39.1-1660.1	Plasma (pg/ml)
el-Kasser *et al* (1998)	N ET RT	28 48 3	245 ±117 287 ±233 123 ±7			Plasma (pg/ml)
	N ET RT	28 48 3	259 ±177 692 ±1129 151 ±17			Serum (pg/ml)
Tahara *et al* (1996)	N(Female) N(Male) ET	21 29 6	0.70 ±0.26 0.79 ±0.35 2.8 ±1.55			Serum (fmol/ml)
Usuki *et al* (1996)	N cMPD	15 12	0.84 ±0.40 1.99 ±1.47			Serum (fmol/ml)
Horikawa *et al* (1997)	N ET	21 17	0.76 ±0.21 1.31 ±1.64		0.22-5.20	Serum (fmol/ml)
Espanol *et al* (1999)	N ET RT	43 15 50	121 ±58 120 ±28 149 ±58			Serum (pg/ml)
Griesshammer *et al* (1998)	N ET	11 7 25	95.3 ±54 545 ±853			Serum (pg/ml)
Hou *et al* (1998)	N ET RT	11 12 13	21.1 ±11.0 44.1 ±45.2 16.4 ±8.6			Plasma (pg/ml)
Tomita *et al* (2000)	N ET	29 59	0.83 ±0.36 2.70 ±2.74			Serum (fmol/ml)
Randi *et al* (2004)	N ET	58 7	25.8 ±14.4 29.6 ±16.1			Plasma (pg/ml)
Wang *et al* (1998)	N ET RT	17 20 34	201 ±112 505 ±459 290 ±133			Serum (pg/ml)

Table 5.1

Circulating TPO levels reported in ET patients

N=normal control, RT=reactive thrombocytosis, cMPD=non-specified myeloproliferative disorder, NEM=non-ET cMPD

studies demonstrated at least a two-fold increase (Griesshammer *et al*, 1998; Hou *et al*, 1998; Abgrall *et al*, 1992; Karakus *et al*, 2002; Pitcher *et al*, 1997; Tahara *et al*, 1996; Tomita *et al*, 2000; Usuki *et al*, 1996; Wang *et al*, 1998). No difference between TPO levels in ET and normal controls was shown in 2 studies (Randi *et al*, 2004; Espanol *et al*, 1999). No difference in TPO levels was seen between males and females (Tahara *et al*, 1996). Only one study investigated both serum and plasma TPO levels. They found that TPO levels in plasma samples from normal controls were 287 ±233 pg/ml, similar to the levels found in plasma samples from ET patients of 245 ±117 pg/ml. However, in serum samples, TPO levels were dramatically increased from 259 ±177 pg/ml in normal controls to 692 ±1129pg/ml in ET patients (el Kassar *et al*, 1998). Differences in TPO levels between serum and plasma may occur because serum samples have platelets removed by clot formation, thus platelet contents can be released and this may affect TPO levels. However, of the 5 studies which used only plasma as the sample material, TPO levels were raised in ET and other cMPDs in comparison to normal controls in 4 studies (Harrison *et al*, 1999c; Hou *et al*, 1998; Karakus *et al*, 2002; Pitcher *et al*, 1997) and were the same as normal controls in only one study (Randi *et al*, 2004). This was similar to the 8 studies which used only serum as the sample material, 7 studies showed raised TPO levels in ET and other cMPDs (Cerutti *et al*, 1997; Espanol *et al*, 1999; Griesshammer *et al*, 1998; Horikawa *et al*, 1997; Tahara *et al*, 1996; Tomita *et al*, 2000; Usuki *et al*, 1996; Wang *et al*, 1998) and only one showed similar levels (Espanol *et al*, 1999) compared to haematologically normal controls. Thus the overwhelming consensus from these studies is that TPO levels are elevated in ET patients compared to normal controls.

However, TPO circulating levels in all categories were very variable. The widest range of values for ET patients was 39.1-1660.1 pg/ml (Pitcher *et al*, 1997), and the mean for ET patients from another study, 692 pg/ml, had a standard deviation of 1129 (el Kassar *et al*, 1998). Thus the range of results obtained for ET patients overlap with that of other groups and many ET patients have higher circulating TPO levels than normal controls.

The studies were also unable to distinguish ET patients from patients with RT or other MPDs. TPO levels in RT were similar to those in ET in three of the studies (Espanol *et al*, 1999; Harrison *et al*, 1999c; Pitcher *et al*, 1997), and while TPO levels were not raised to the same degree as in ET patients in two further studies, they were raised compared to normal controls (el Kassar *et al*, 1998; Wang *et al*, 1998). TPO levels in other MPDs were also equal or raised compared to haematologically normal controls as

demonstrated by the studies which did not separate ET from other MPDs (Karakus *et al*, 2002; Cerutti *et al*, 1997; Usuki *et al*, 1996). The increase in TPO circulating levels in these studies were similar to increases seen in ET patient groups.

Whilst circulating levels of TPO cannot be used diagnostically in ET because of the large overlap between cMPD, RT and normal cohorts, the question as to why TPO levels are high remains. The sponge theory suggests that the increased platelet mass of the thrombocytosis should remove TPO from the circulation leading to subnormal TPO levels, but this is not the case in ET. The high level of TPO may be caused by up-regulation of factors which modulate TPO expression, and this would be important for ET pathogenesis.

5.1.6 Hereditary Thrombocytosis Studies

Hereditary thrombocytosis (HT) is an inherited condition which shares many features with ET. Patients with HT fulfil the diagnostic criteria for ET laid down by the PVSG (Ghilardi & Skoda, 1999). Both diseases are characterised by elevated platelet counts due to sustained over-proliferation of MKs, and frequently lead to thrombotic and haemorrhagic complications. HT is often inherited in an autosomal dominant fashion, although one family has been identified in which inheritance is probably in an X-linked fashion (Stuhrmann *et al*, 2001). To date, four point mutations have been identified in the 5'UTR of the TPO gene in HT families. All four mutations lead to a reduction in the suppression of transcription initiation from AUG 7.

The first mutation described in the TPO 5'UTR was a G to C substitution at nucleotide 3916 (Wiestner *et al*, 1998). It was identified in a cohort of four generations of the affected family. All affected individuals had elevated platelet counts (880-1280x10^9/L) and serum TPO levels (74-1180pg/ml) compared to unaffected family members (220-330 x10^9/L and <62pg/ml respectively). The mutation was present in all affected individuals and was shown to disrupt the splice donor site of intron 3 by replacing the 5' G with a C. The splice donor site on intron 4 was used instead and an mRNA transcript missing exon 3 was produced (Figure 5.2A). This led to removal of the two most significant start sites, AUG 7 and 8, from the transcript, and brought AUG 5 and 6 into frame with the normal coding sequence. Thus, the suppressive effect on translation from AUGs 5, 6 and 7 was removed, but functional protein is still produced. A TPO construct containing the mutation was transfected into a rat hepatoma cell line (HTC). TPO levels in supernatants taken from

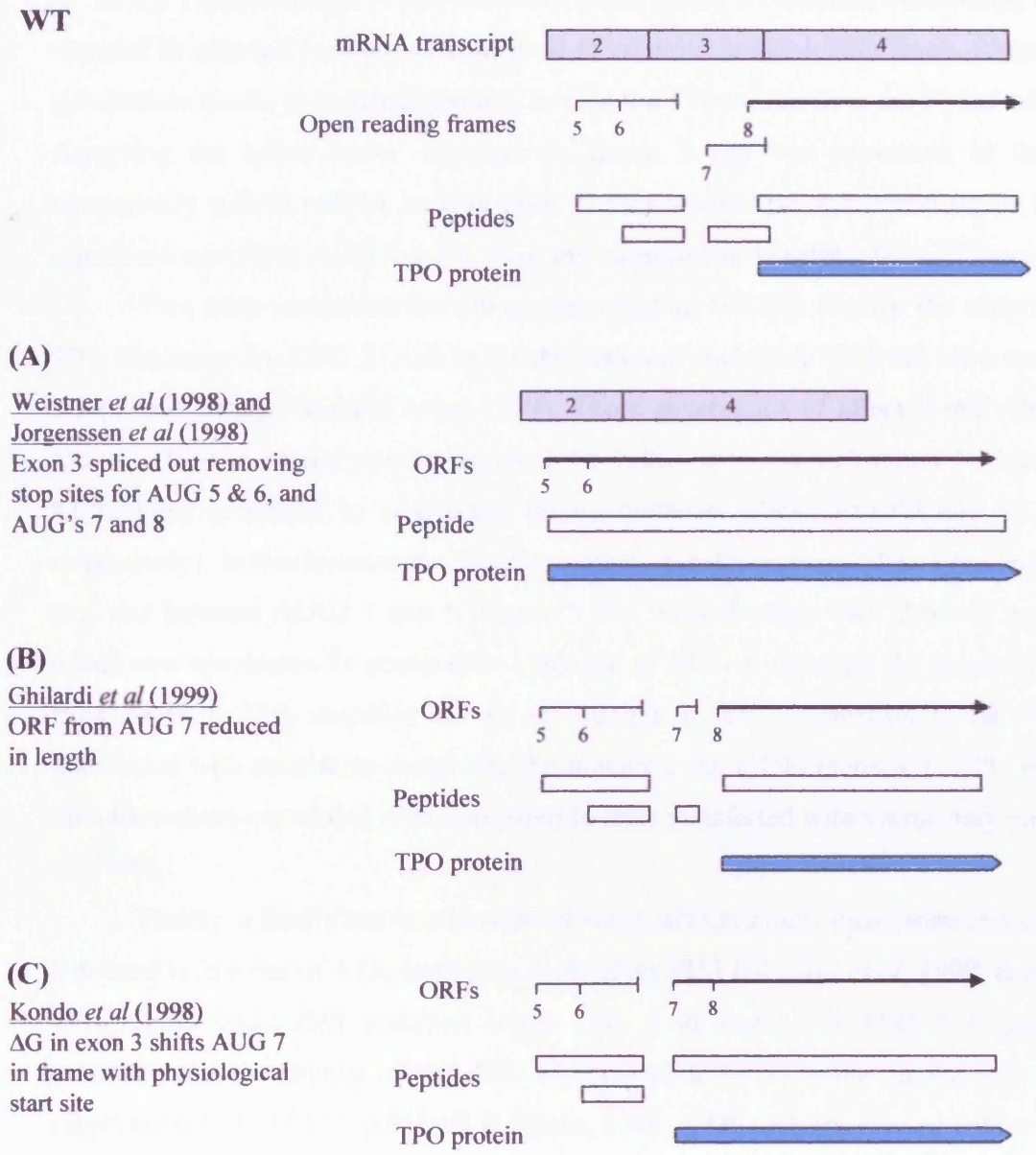

Figure 5.2
Open Reading Frames produced from TPO mRNA for each of the HT mutations

The mRNA structure is shown in grey with exon numbers marked. Start sites (AUG) are numbered in open reading frames (ORFs) and stop sites are indicated by a vertical bar. Functional TPO protein is indicated with blue shading.

these cell lines were significantly increased (210±6pg/ml) compared to cells transfected with vector only and untransfected controls (<62pg/ml). A G to A substitution at nucleotide 3920 was described in all four affected members of another family with HT (Jorgensen *et al*, 1998). Platelet counts (700-1000x10^9/L) and serum TPO levels (430-980pg/ml) were elevated in affected individuals compared to controls (control TPO levels 85pg/ml). The substitution results in a change from G to C at the 5th position from the 5' end of intron 3, disrupting the splice donor sequence in intron 3 and was presumed to lead to an alternatively spliced mRNA lacking exon 3. This would lead to removal of the two most significant start sites, AUG 7 and 8, from the transcript as described above (Figure 5.2A).

Two other mutations have been described in HT that remove the suppression on TPO translation by AUG 7. A G to T substitution at nucleotide 3872 has been described in a Japanese family (Ghilardi *et al*, 1999). Three generations of affected individuals were shown to have increased platelet counts (1095-1380x10^9/l) and high serum TPO levels (5.6-8.0fmol/ml) compared to unaffected family members (189-274x10^9/l and <1.3fmol/ml respectively). In this instance the mutation affects translation from AUG 7 by introducing a stop site between AUGs 7 and 8 (Figure 5.2B). This shortens ORF 7 by 42 nucleotides, which now terminates 31 nucleotides upstream of AUG 8, reducing the suppressive effect from AUG 7. This mutation led to an increase in TPO expression in rat HTC cells transfected with constructs containing the mutation. An 8-fold increase in TPO levels was seen for mutant-containing cells compared to cells transfected with vector only (Ghilardi *et al*, 1999).

Finally, a family has been described where affected individuals were shown to carry a deleted G in a run of 4 Gs beginning at position 3853 (Ghilardi *et al*, 1999; Kondo *et al*, 1998). This frame shift mutation brings ORF 7 in frame with ORF 8 (Figure 5.2C), extending the N-terminus of the TPO signal peptide by 23 amino acids, and removing suppression from AUG 7 (Ghilardi & Skoda, 1999). COS cells transfected with a construct containing the mutation secreted 7-fold more TPO than cells transfected with wild type TPO. This indicated that not only does the mutation improve efficiency of TPO translation, but also that the alteration to the signal peptide does not prevent secretion of TPO protein.

The 4 mutations appear solely responsible for the increase in circulating TPO levels associated with the HT disease phenotype in the families studied. Since the phenotype of ET matches that of HT it is possible that some ET patients may have acquired one of the described mutations, or a similar alteration with the same effect on suppression of TPO

translation. This may be more significant in polyclonal patients where there is no clonal expansion of a megakaryocytic progenitor and factors derived from outside the MK progenitor could be of greater importance. It has been demonstrated that the G3916C mutation, which leads to the exon skipping of exon 3 and removal of AUGs 7 and 8, was not present in 51 ET patients (Harrison *et al*, 1998b). However, none of the other mutations have yet been examined in ET patients, nor have novel mutations been sought.

5.1.7 AIM

The aim of the work detailed in this chapter was to establish whether any of the three HT mutations, not yet investigated in ET, were present in a cohort of 50 ET patients using restriction enzyme analysis, and to analyse for any novel mutations in the TPO 5'UTR which may affect TPO expression levels using PCR-SSCP.

5.2 Materials and methods

5.2.1 Patients and Samples

Peripheral blood samples were collected from 50 female patients with a diagnosis of ET according to the PVSG criteria and 100 haematologically normal controls. Patient characteristics are outlined in Table 5.2. DNA was prepared from purified neutrophils as detailed in chapter 2 (2.2.1 and 2.2.2).

5.2.2 PCR

Approximately 100ng of DNA was used for 30 cycles of PCR using Bioline reagents as outlined in chapter 2 (2.2.7). The primers and annealing temperatures used are listed in Table 5.3.

5.2.3 Analysis of known HT mutations

PCR was carried out on DNA from patient neutrophils as outline above. The G3872T substitution (Ghilardi *et al*, 1999) was investigated with *BslI* digestion of 3F/3R PCR products. The full length PCR product of 365bp was digested into fragments of 28, 149 and 188bp for WT alleles and 149 and 216bp for mutant alleles (Figure 5.3A). The G3920A mutation (Jorgensen *et al*, 1998) was investigated with primer 3F and a mismatch primer MM3920 followed by digestion with *MboI* (Figure 5.3B), which would give fragments of 28 and 227bp for WT alleles and remain undigested for mutant alleles (255bp). The ΔG mutation at nucleotide 3853 (Kondo *et al*, 1998) was investigated using mismatch primers MM3852 and MM3920 to allow a suitable size PCR product for digestion. *SacII* digestion would give fragments of 29 and 203bp for WT alleles and mutant alleles remain undigested (232bp) (Figure 5.3C).

5.2.4 Single Stranded Conformational Polymorphism (SSCP) Analysis

As the size of the 5'UTR of the TPO gene is large (over 1200bp), and 50 patients being investigated, direct sequencing was considered too impractical and expensive to be used as a method of mutation detection. To detect sequence changes quickly and efficiently a screening method was used. SSCP is a relatively rapid screening technique sensitive enough to detect single base pair substitutions and previously has been used successfully in

Age at Diagnosis (years)	Median = 53.5	Range 10-89
Platelet Count at Diagnosis($x10^9/l$)	Median = 837	Range 600-1917
Follow up at Test (months)	Median = 38	Range 0-240
Karyotype	Normal	27
	+8, +9	1
	20q-	1
	Not known	21
Clinical Symptoms at Test	Hepatosplenomegaly	12
	Haemorrhage	6
	Thrombosis	22
	None	20
Treatment at Test	^{32}P	2
	Chemotherapy*	34
	None	16
Clonality	Monoclonal	10
	Polyclonal	15
	Uninterpretable	22
	Not Tested	3

Table 5.2
Clinical details of the 50 ET patients
*includes Busulphan, Hydroxyurea, Interferon-α, Anagrelide

Fragment	Forward primer (5'→3')	Reverse primer (5'→3')	Nucleotides*	Fragment Size (bp)	Annealing Temp (°C)
1	GTCGGTGTCCAGCCCAGGAAG	CACTGCCTAGCCTGCCTCCCT	428-654	226	64
2A	CCTCTGTGCTTCTTCCCCAGC	GTATGACCTGCTGCTGTGGAG	1702-1992	290	60
2B	GTGTGTGTGGGTGGAGGAGTG	AGAAATGGGCTCCCAGCTGGG	1841-2158	317	60
3	ACCCTGCCAGGCAGTCTCTTC	GAGGGGTGGATTCCCTGGGTT	3695-4057	362	60
4	AGAGAAAGGAGACACGCTGCA	TCCGCGTAACTGGTAAGACAC	3969-4323	354	60
MM3920†	ATCACACCTGAGGGGCTAGGGCCATATGGA (Down Primer)		3768-3797		64
MM3852†	CGCCTCCATGGCCCCAGGAAGGATTCCGCG (Up Primer)		3921-3951		64
MM3767†	TCAGAATTGGCCCGCCTTTGCCCCACCCTA (Down Primer)		3826-3855		68

† Mismatch primers with mismatched bases underlined
* Numbered according to Chang *et al* 1995

Table 5.3
PCR primers used to investigate the 5'UTR of the TPO gene
The PCR fragment sizes and the annealing temperature used for each primer pair are also shown

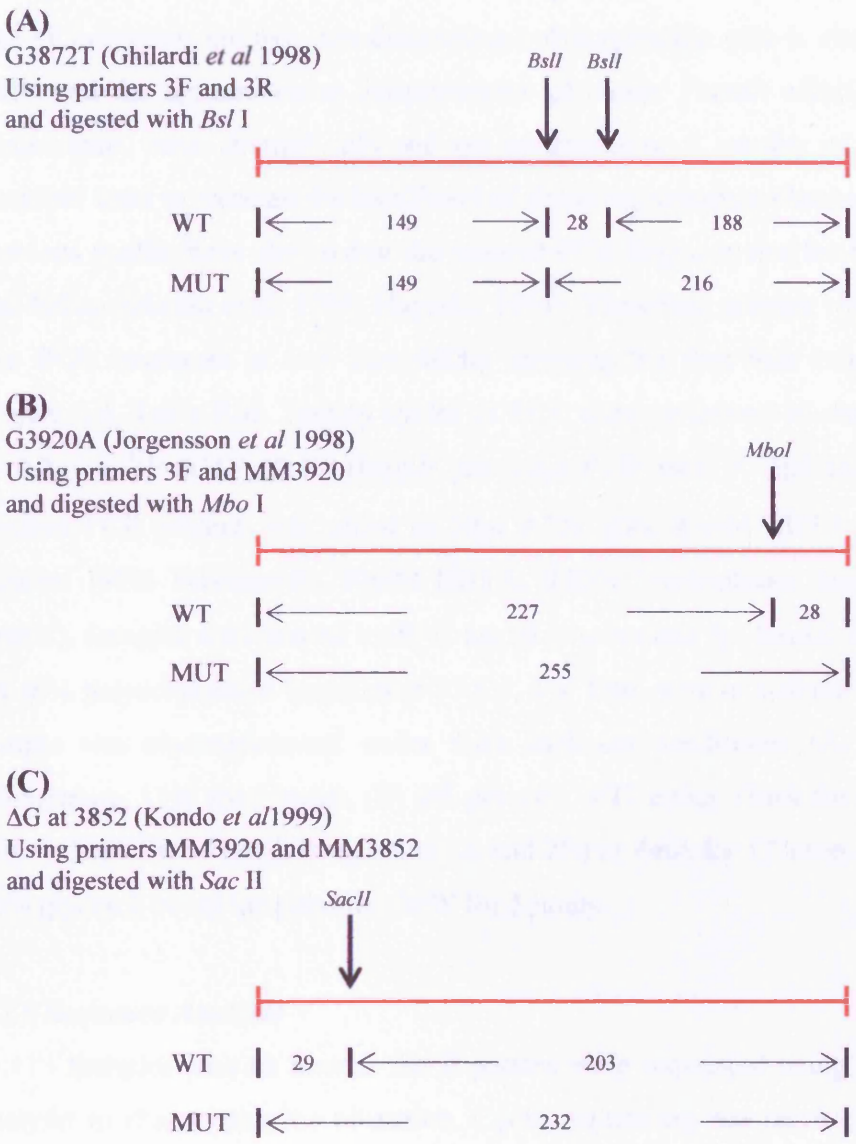

Figure 5.3
PCR and restriction enzyme digests for each of the HT mutations studied.
Fragment lengths produced by RE digestion of PCR products for each of the HT mutations investigated in ET patients. PCR primers and enzymes used for each digest are detailed. Fragment sizes are given in bp.

the laboratory. Under non-denaturing conditions single stranded DNA has a folded conformation dependent on intra-strand interactions, and the folding is sequence specific. Changes to the sequence thus influence folding and, in suitable experimental conditions, the rate of migration through non-denaturing polyacrylamide gels is altered leading to band shifts and the appearance or disappearance of bands. Factors affecting mobility include temperature, ionic strength, pH and gel composition. A number of gel conditions were therefore used to increase the likelihood of detecting sequence changes, as outlined below. Previous studies have shown that the optimal PCR fragment size for SSCP is between 200 and 400bp (Glavac *et al*, 1994; Hayashi, 1991). Therefore, primers were designed to create five PCR fragments of less than 400bp covering the first four exons of the TPO gene (Figure 5.4, Table 5.3). Twenty cycles of PCR were performed as above with the addition of 0.2µl α-^{32}P-dCTP (0.37MBq/µl) per 20µl PCR mix. A 2µl aliquot of radioisotope labelled PCR product was added to 10µl 0.1% SDS, 10mM EDTA and 11µl denaturing solution (95% Formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). Samples were heated to 95°C for 10 minutes and 5µl loaded onto a non-denaturing gel (6% polyacrylamide crosslinker 37.5:1, 1 x TBE, with or without 10% glycerol). Each sample was electrophoresed under three different conditions: (A) 0% glycerol, room temperature, 15W for 5 hours, (B) 0% glycerol, 4°C, either 23mA for 4 hours (fragments 3 and 4), 5mA for 17 hours (fragments 2A and 2B) or 4mA for 17 hours (fragment 1) and (C) 10% glycerol, room temperature, 30W for 5 hours.

5.2.5 Sequence Analysis

Samples with an altered SSCP pattern were sequenced using the ABI 310 genetic analyser to characterise the alteration. Cycle sequencing was carried out using BigDye™ Terminator Cycle Sequencing Ready Reaction, 310 Genetic Analyser Kit, as outlined in Chapter 2 (2.2.9) in both the 5' and 3' direction. Sequence alterations were then confirmed using restriction enzyme digestion.

5.2.6 Mutation-sensitive RE analysis

PCR products were digested as described in chapter 2 (2.2.11), electrophoresed through agarose gels and visualized by ethidium bromide staining.

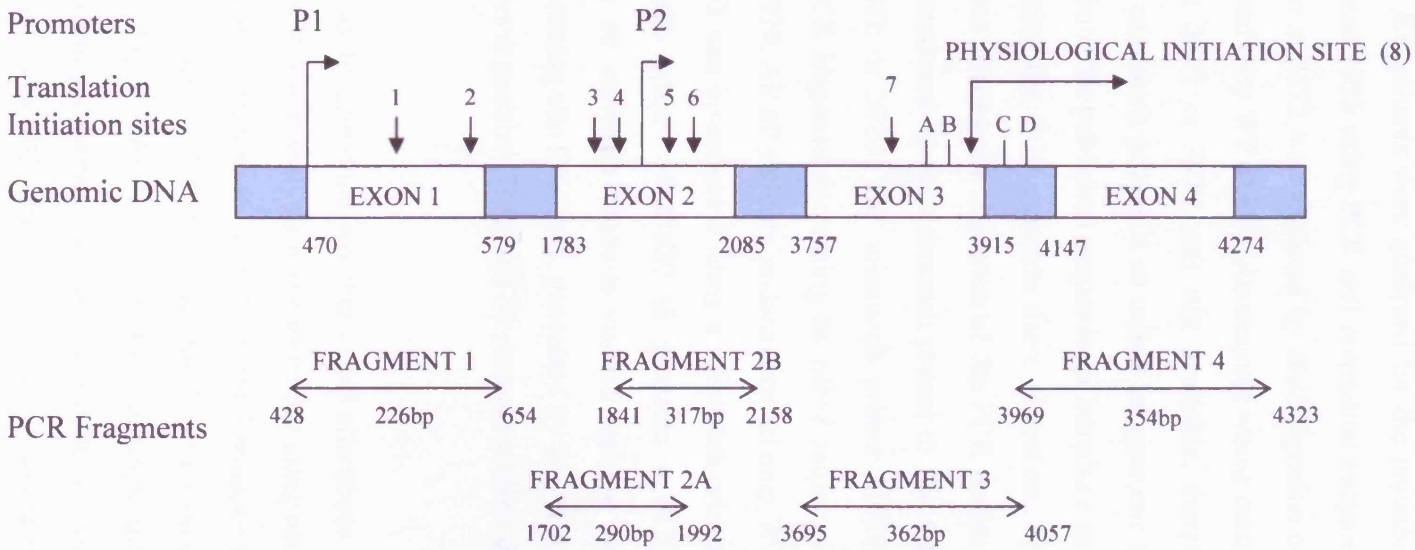

Figure 5.4

PCR-SSCP fragments used to analyse the 5'UTR of the TPO gene

The PCR fragment sizes (bp) and position within the gene are shown (numbered according to Chang *et al* 1995). The positions of the 8 translation initiation sites (1-8) and the mutations described in HT (A,B,C and D) are also illustrated.

5.3 Results

5.3.1 Analysis of known HT mutations

Fifty ET patients were analysed for the mutations described in HT at nucleotides 3852, 3872 and 3920 using PCR and restriction enzyme digestion (Figure 5.3, Table 5.3). The mutation at 3872 was analysed by *BslI* digestion of PCR fragment 3. All 50 patients studied showed only WT alleles. An enzyme whose cutting site enabled the detection of the mutations at 3852 or 3920 was not available, therefore two mismatch primers were designed. A mismatch primer is so called because one or more of the primers nucleotides are altered from the published sequence to introduce an enzyme-cutting site into the PCR product. During the PCR reaction these alterations are introduced into every fragment produced, thus altering the sequence of the PCR product. In this instance, enzyme-cutting sites were introduced by the mismatch primer to allow the detection of wild type alleles at positions 3852 or 3920. The mismatch primer MM3920 was used with primer 3F to produce a PCR fragment containing an *MboI* cutting site which would cut WT alleles at nucleotide 3920. All 50 patients studied showed only WT alleles. Similarly, the mutation at position 3852 was investigated using a mismatch primer, MM3852. This primer was used with mismatch primer MM3920 to generate a PCR product of a suitable size for visualisation on ethidium bromide stained agarose gels. The PCR fragment produced contained a cutting site for *Sac*II, generated by the MM3852 mismatch primer, that would cut WT alleles at position 3852. All 50 patients studied showed only WT alleles.

5.3.2 SSCP

In order to determine whether novel alterations were present in the 5'UTR of the TPO gene, PCR-SSCP analysis was carried out using neutrophil DNA from 50 ET patients, and the patterns compared to DNA from the haemopoietic cell lines HL60 and TF-1. Five PCR fragments were designed to cover the first four exons and flanking regions of the TPO gene (Figure 5.4). The fragments therefore encompassed the 5'UTR and the first 47 amino acids of the coding sequence. No abnormal patterns were detected in fragments 1, 2A, 2B or 4. However, three different patterns were observed in fragment 3 (Figure 5.5A). In 12 patients and the HL60 control, pattern 1 was observed in condition C; three equally spaced bands were seen with the upper two bands more intense than the third lower band. Fourteen

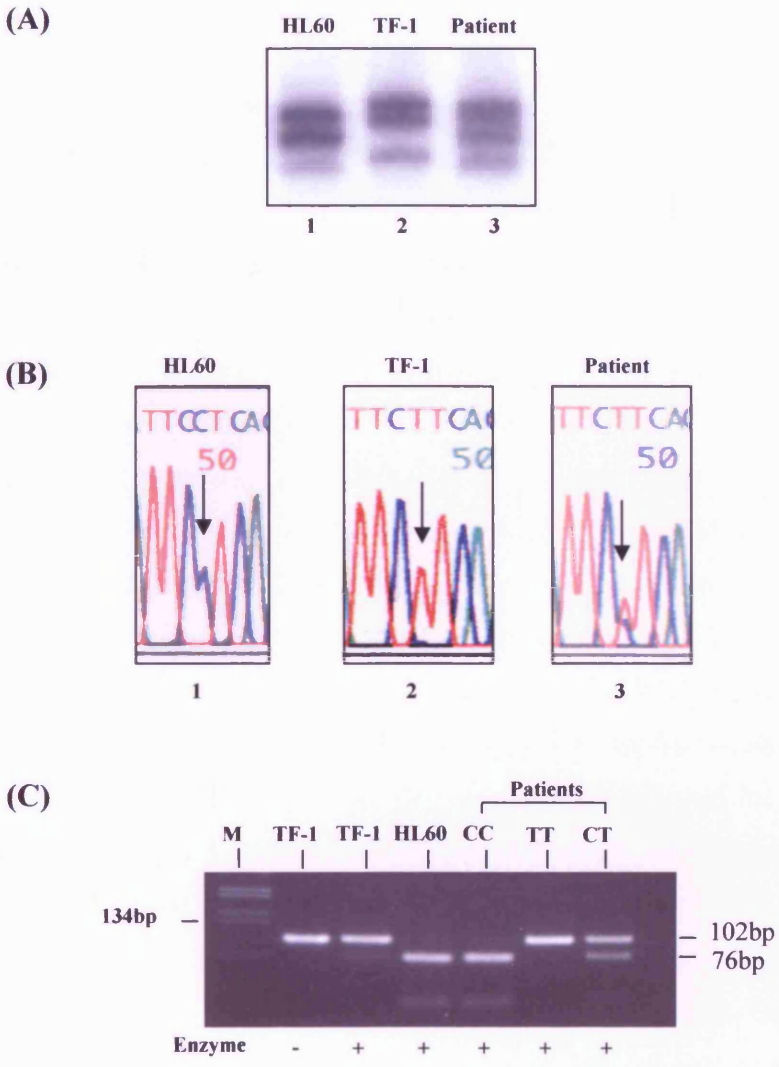

Figure 5.5
Analysis of the alteration at nucleotide 3767 in the 5'UTR of the TPO gene.
A: PCR-SSCP patterns for each genotype
B: Sequencing results for each genotype with arrow indicating nucleotide 3767
C: *Dde I* digestion to identify C3767T polymorphism.

patients and the TF-1 control shared pattern 2; three bands could again be seen, two with a higher intensity than a lower third band. However, the mobility of each band was slightly different when compared to pattern 1. Pattern 3 was found in 24 patients; the bands were less clearly resolved, but appeared to contain all the bands present in patterns 1 and 2.

5.3.3 Sequencing and Genotyping

A representative sample of each PCR-SSCP pattern described above was sequenced to characterise the sequence alteration. As shown in Figure 5.5B, an alteration to the published genomic sequence was identified at position 3767 in two of the samples. The published sequence carries a C at this position. HL-60 was found to be homozygous for C at 3767, TF-1 was shown to be homozygous for T, and the patient sample with SSCP pattern 3 was heterozygous CT.

A mismatch primer MM3767 (Table 5.3) was created which introduced a *Dde*I cutting site in T-containing alleles. Digestion of 3F/MM3767 PCR products confirmed results for the patients, HL60 and TF-1. C-containing alleles remained uncut at 102bp, T-containing alleles digested to 76 and 26bp (Figure 5.5C). A complete correlation was shown between digest pattern and SSCP pattern in the ET patients. To establish whether this alteration was a common polymorphism, or a mutation possibly relating to ET, 100 haematologically normal control samples were also investigated for the alteration at 3767. The genotypes of the ET group were 24% homozygous C, 48% heterozygous CT, and 28% homozygous T. The genotypes for normal controls were 20% homozygous C, 52% heterozygous CT and 28% homozygous T. The allele frequency for C at position 3767 was 0.48 and 0.46 for ET patients and normal controls respectively. There was no significant difference between ET patients and the cohort of 100 controls at either the genotypic or allele frequency levels. Twenty five of the ET patients were interpretable for clonality status by XCIP, 10 had clonal myelopoiesis and 15 had polyclonal myelopoiesis. Of the 10 clonal patients, 30% were C homozygous, 60% heterozygous CT, and 10% were homozygous T. The genotypes of the 15 polyclonal patients were 40% homozygous C, 47% heterozygous CT, and 13% homozygous T (Figure 5.6). These data gave an allele frequency for the C allele of 0.60 for clonal patients and 0.64 for polyclonal patients. No correlation between clonality status and genotype at position 3767 could be established ($p>0.5$). There was also no significant difference between the clonal patients and normal controls ($p>0.4$) nor between the polyclonal patients and normal controls ($p=0.1$) at the

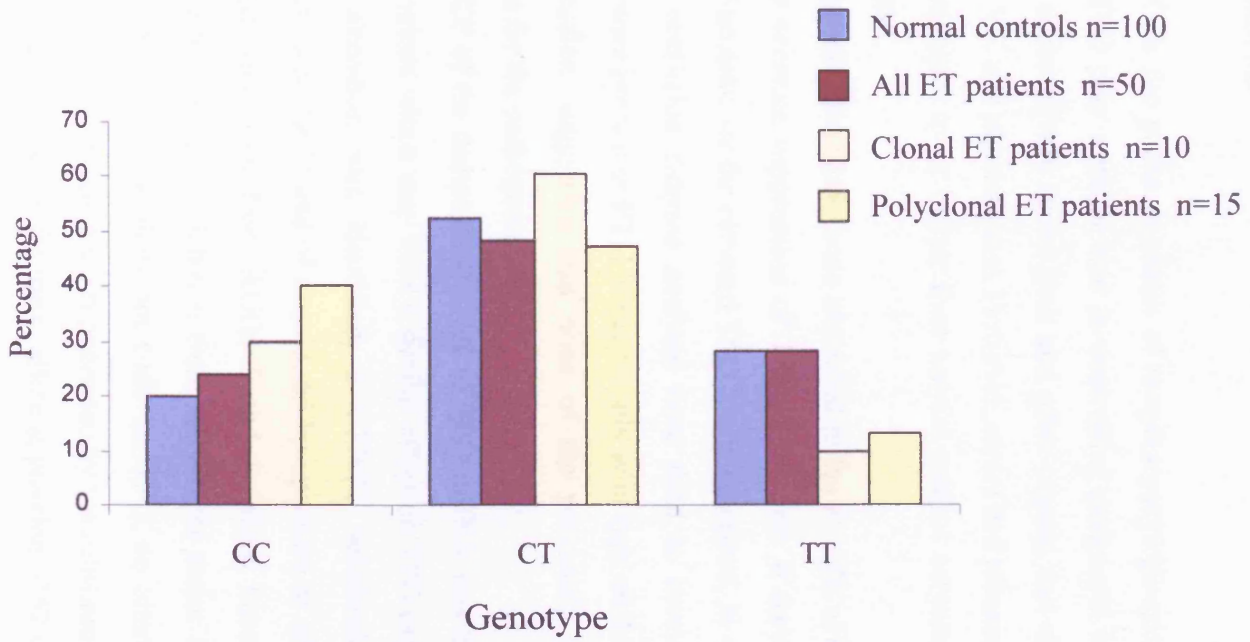

Figure 5.6
The genotypes at nucleotide 3767 of the TPO gene in ET patients and normal controls

genotypic level. The small differences observed are probably representative of the small number of patients studied.

5.4 Discussion

TPO is the prime regulator of megakaryocytopoiesis, and as such the circulating levels of TPO play a major role in controlling peripheral blood platelet counts. TPO is expressed constitutively in the liver and other organs, and circulating levels are regulated mainly by MK and platelet mass. However, serum and plasma concentrations of TPO in ET patients are equal to or higher than normal control subjects, in spite of the increase in platelet mass.

Four mutations have been identified in the 5' UTR of the TPO gene in families with HT, which decrease suppression of TPO translation. If they were present in ET patients they could account for the elevated TPO levels described, as well as the disease phenotype. PCR and restriction enzyme analysis were used to investigate whether any of these mutations were present in ET. However, only wild type alleles were identified in the 50 ET patients studied, suggesting that none of the previously described HT mutations are responsible for the pathogenesis ET.

SSCP of the complete 5' UTR of TPO mRNA was then carried out to analyse for novel alterations which may have a similar effect on TPO expression as the HT mutations. A single alteration was identified, a C3767T substitution. The alteration is 10bp downstream from the 5' end of exon 3, 65 bp upstream of AUG 7. While this substitution could affect translation from AUGs 5 and 6, which have been shown to have some inhibitory effect *in vitro*, inhibition from AUG7, the major inhibitory start site, would be unaffected. As the region does not code for TPO, no alteration to its protein sequence occurs. No start or stop sequence is generated by the substitution.

An allele frequency for the C allele at position 3767 of 0.48 was determined in ET patients and 0.46 for haematologically normal control subjects. The difference between the two groups was not statistically significant and it is therefore unlikely that either allele alone has any effect on disease pathogenesis. At the genotypic level, no significant differences could be demonstrated. The genotype CC was present in 28% of patients, CT

48% and TT 28%. Normal control genotypes were similar at CC 20%, CT 52% and TT 28% and again no significant differences could be shown. Only 25 patients in the group had known clonality status, and although there were some small differences probably due to small sample size, there was no significant bias towards a particular allele frequency or genotype in either clonal or polyclonal patients. The different alleles at this locus are therefore unlikely to have any effect on efficiency of TPO translation and are probably a naturally occurring single nucleotide polymorphism with no affect on disease pathogenesis.

The lack of acquired or inherited mutations in the 5'UTR of TPO in ET patients does not rule out the presence of mutations or polymorphisms in the coding region of the TPO gene. Genetic alterations may affect TPO transcript or protein stability which could lead to an increase in circulating levels if TPO is less readily degraded. Also, alterations affecting the binding efficiency of TPO to Mpl could lead to less efficient removal from the circulation leading to an abnormally high TPO level. However, alterations to the coding sequence of TPO have yet to be investigated in ET.

Two promoters in the TPO gene, P1 and P2, give rise to 2 mRNA isoforms by alternative promoter usage. A full length isoform is produce from P1 and a truncated mRNA, missing exon 1 and part of exon 2, is produced from P2. The isoform produced from P2 is more efficiently translated than the full-length transcript due to the absence of AUGs 1-4 and the subsequent loss of their suppressive effect on translation. In liver, the major site of TPO production, 90% of transcripts are generated from P2 (Ghilardi & Skoda, 1999). A increase in promoter P2 usage in ET could lead to a small, but significant increase in TPO expression. Also, promoter usage may vary between cell types which may be important for local effects. If promoter P2 usage is less in liver than in BM stromal cells then TPO would be less efficiently expressed in BM. An increase in the proportion of TPO transcripts generated from P2 in ET patients may than lead to a large increase in BM TPO levels. However, as liver biopsies are not taken in ET patients and BM samples are difficult to obtain, studies of isoform usage in ET are difficult.

Other isoforms affecting TPO at the protein level may contribute to the pathogenesis of ET. Six different TPO protein isoforms have been identified in carcinoma cell lines: the full length TPO mRNA, four deletions in exon 6 of sizes 12bp, 116bp, 128bp and 197bp (TPO-1 to TPO-5 respectively) and a 60bp insertion of intron 5 coupled with the 116bp deletion of exon 6 (TPO-6). Twenty seven carcinoma cell lines were tested and 24 were found to contain TPO-1 mRNA with one or more of the other isoforms. The

biological activities of TPO-5 and TPO-6 were studied and neither was able to promote the proliferation of TPO-responsive FDCP-hMp15 cells, whereas TPO-1 was able to, in a dose-dependent fashion. These non-biologically active isoforms could compete with TPO-1 for access to the TPO receptor, possibly preventing TPO internalisation and subsequent degradation. While this would not be expected to lead to a proliferative or survival advantage for the MK lineage, it may lead to an increase in circulating TPO levels secondary to another underlying defect.

While the mutations described in the families with HT were causative of their disease, not all HT patients carry mutations in the 5'UTR of their TPO gene. Two reports document two families where TPO mutations were not present. Affected individuals had normal TPO circulating levels, compared to raised levels in the families with TPO mutations. Linkage analysis was used to exclude TPO in one family, direct sequencing in the other (Wiestner *et al*, 2000; Tonelli *et al*, 2000). As TPO is not involved in these two families, other factors must be contributing to the disease phenotype.

One such candidate is Mpl. TPO binds to Mpl on the surface of MKs and platelets causing downstream signalling events as outlined in chapter 1. Once binding has occurred, the receptor, with TPO still bound, is internalised and TPO degraded (Fielder *et al*, 1997). Levels of Mpl in the HT family carrying the C to G mutation at nucleotide 3916 were decreased in 7 out of 8 affected individuals, despite having normal mRNA expression of c-mpl (Kralovics *et al*, 2003), and there is increasing evidence that Mpl levels are reduced in ET patients.

A marked reduction of the level of Mpl on the surface of platelets, and a reduction in total Mpl protein was seen in 14 ET patients compared to 5 normal controls using flow cytometry and western blot analysis (Horikawa *et al*, 1997). A decrease in the level of c-mpl mRNA was also shown by northern blot and semi-quantitative PCR. A significant decrease in the surface expression of Mpl on 18 ET platelets was demonstrated using ^{125}I-labelled antibodies bound to purified platelet preparations (Harrison *et al*, 1999c). Surface c-mpl levels in ET patients ranged from 0%-63% of control values ($p<0.001$). However, while a significant difference in c-mpl surface expression was shown between these ET patients and 8 RT patients ($p=0.0015$), no significant difference could be shown between the ET cohort and 8 patients with other MPDs, nor did Mpl surface expression differentiate patients with clonal or polyclonal haemopoiesis. A decrease in protein levels was also shown by western blotting.

A similar study using flow cytometry showed that there was a 10 fold decrease in the number of TPO binding sites on platelets from 23 ET patients compared to 19 normal controls (Li *et al*, 2000). ET patients had 5.6 ±5.5 TPO binding sites per platelet and normal controls 56±17. From this data the clearance rate of TPO from the circulation could be established as 0.30±0.14ml/hr/10^9 platelets in ET patients compared to 1.24±0.38ml/hr/10^9 platelets in normal controls.

Immunohistochemical studies have also show a reduction in Mpl levels in ET patients compared to control samples, and this is combined with a more heterogeneous staining pattern than controls (Duensing *et al*, 1999; Teofili *et al*, 2002b; Yoon *et al*, 2000). The decrease in platelet c-mpl expression could lead to platelet c-mpl becoming saturated, preventing further uptake of TPO from the circulation, and this may account for the rise in circulating TPO levels seen in ET.

Mutations in the c-mpl gene may also lead to a decrease in TPO clearance from the circulation by preventing c-mpl translocation to the cell surface, disrupting TPO binding or preventing TPO internalisation. This may result in elevated circulating levels. A large proportion of congenital amegakaryocytic thrombocytopenia (CAMT) patients have a mutation in the c-mpl gene. A 10-year-old girl with CAMT was described who had inherited two c-mpl mutations, one from each parent (Ihara *et al*, 1999). Each mutation caused truncation of the Mpl protein to produce a peptide lacking all intracellular domains essential for signal transduction. Another study analysed the c-mpl sequence of 5 CAMT children and found that 4 of the 5 patients had c-mpl mutations, 3 caused an amino acid change or led to a premature stop codon, while the fourth resulted in loss of a splice site (van den Oudenrijn S. *et al*, 2000). Another study found c-mpl mutations in 8 out of the 8 CAMT patients they studied (Germeshausen *et al*, 2001).

A single nucleotide polymorphism, a G to T at nucleotide 1238 of the c-mpl gene, has been identified in approximately 7% of African Americans which changes a lysine to an asparagine at codon 39 (Moliterno *et al*, 2004). Individuals with an asparagine at codon 39 had a slight, but significant thrombocytosis ($p<0.001$). This was associated with a marked decrease in c-mpl expression in two patients, as assayed by western blot and Q-RT-PCR of 32D cells transformed with mutant or wild type c-mpl. However, circulating TPO levels were not investigated in the study.

A c-mpl mutation has also been identified in a family with HT (Ding *et al*, 2004). The activating point mutation, G to A at nucleotide 1073 of the c-mpl gene was identified

in 8 affected family members, but was not present in 8 unaffected family members. The mutation led to replacement of a serine residue at codon 505 with an asparagine. It was shown to confer IL-3 independence on Ba/F3 cells transfected with the mutant c-mpl, and random mutagenesis had previously shown that this mutation led to an increase in sensitivity to TPO in mice (Onishi *et al*, 1996). However, none of the 19 ET patients screened had the mutation.

Mutations in the c-mpl gene have not yet been described in ET patients. Two alterations to the c-mpl sequence were found in 2 out of 4 ET patients sequenced in one study. However, these were also found to be present in normal controls and assumed to be common polymorphisms (Kiladjian *et al*, 1997). A second study sequenced the whole coding region of c-mpl in 9 ET patients and found no alterations (Taksin *et al*, 1999). Another study detailed 7 young ET patients where no alterations to the c-mpl gene could be identified by direct sequencing (Randi *et al*, 2004). The number of ET patients investigated for c-mpl mutations is still small, so mutations to c-mpl in ET patients cannot be ruled out. However, as disease-specific mutations have not been identified in the 20 ET patients studied so far, it is unlikely that they are a major factor in the pathogenesis of ET.

In conclusion, the increased or normal circulating TPO levels in ET patients is not due to mutations in the 5' UTR of the TPO gene. The evidence for the involvement of disregulated Mpl expression in the high serum/plasma TPO levels is strong, but further study of TPO regulation is necessary to determine whether altered TPO levels can be implicated in the pathogenesis of ET.

Chapter 6

Polymorphisms in the TGFβ1 and IL-6 genes in essential thrombocythaemia patients

6.1 Introduction

6.1.1 Transforming growth factor β1

The transforming growth factor family of proteins, originally isolated from Maloney MuSV transformed 3T3 fibroblasts, are involved in most processes concerned with somatic tissue development and renewal (De Larco & Todaro, 1978). Virtually every cell in the body, including haemopoietic cells, produces and has receptors for TGFβ, although the main storage site of TGFβ1 is in the α-granules of MKs and platelets (Assoian *et al*, 1983). In-situ hybridization using a TGFβ1 specific probe has demonstrated the presence of TGFβ1 mRNA in MKs indicating that TGFβ1 is synthesised in MKs, and immunohistochemical studies have directly shown storage of TGFβ1 in α-granules (Fava *et al*, 1990). Three highly similar isoforms of TGFβ have been identified in mammals, TGFβI, II and III (de Martin *et al*, 1987; Derynck *et al*, 1985; ten Dijke *et al*, 1988), and while the most studied isoform in haemopoietic tissue is TGFβ1, all three isoforms have some involvement in the regulation of haemopoiesis.

TGFβ is generated by the production of a primary translation product (Figure 6.1), which consists of three regions, a pre region (αα1-29) which forms a short signal peptide, a pro region (αα30-278) and active TGFβ (αα279-390) (Gentry *et al*, 1988). The pro region, once cleaved, forms a homodimer termed latency associated protein (LAP) which non-covalently binds with TGFβ dimers to form the 'small complex'. This small complex therefore contains 'latent' TGFβ which is prevented from reaching target cells (Miyazono *et al*, 1988). Latent TGFβ binding proteins (LTBP) then bind the small complex to form the large complex, which can associate with the extra cellular matrix and act as a store of readily available TGFβ (Wakefield *et al*, 1988).

Release of latent TGFβ may occur *in vivo* by proteolytic cleavage by plasmin or transglutaminase, or conformational modification of LAP by thrombospondin. The *in vitro* release of latent TGFβ is achieved by acidification, and therefore measured TGFβ levels are often quoted as a combination of active and acid inducible latent TGFβ ((a+l) TGFβ).

Once released, TGFβ binds to TGFβ receptor II which then recruits TGFβ receptor I, both of which are serine/threonine kinase receptor family members. This leads to formation of a protein complex at the cell surface containing one TGFβ dimer bound to a

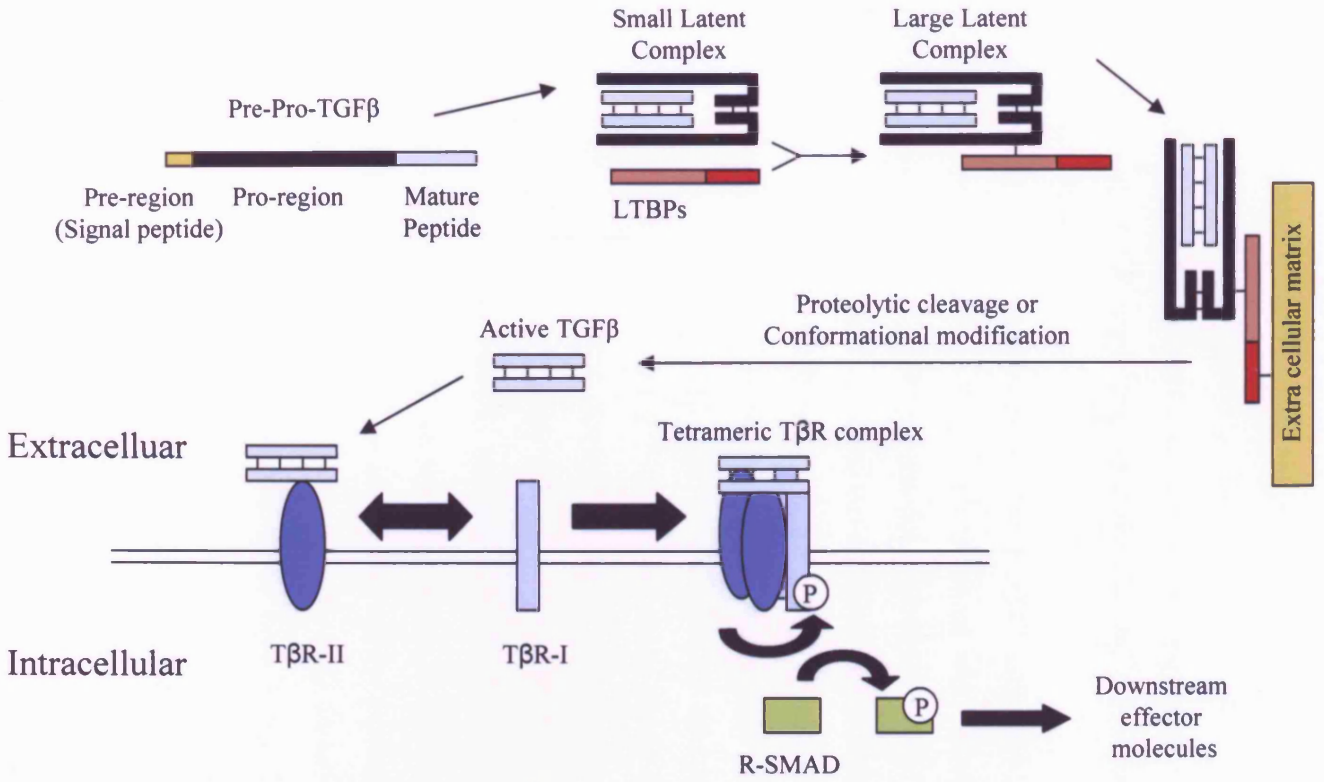

Figure 6.1

TGFβ structure, latency and signal transduction

Diagram showing the processing of the primary TGFβ peptide to form latent TGFβ in association with latent TGFβ binding proteins (LTBPs), release of active TGFβ, and receptor binding leading to signal transduction. TβR = TGFβ receptor (Modified from Fortunel *et al* 2000)

tetrameric TGFβ receptor complex consisting of two TGFβ receptor I and two TGFβ receptor II molecules (Wrana *et al*, 1994). TGFβ receptor I is then phosphorylated by the constitutively active TGFβ receptor II and downstream signalling occurs via the Smad family of proteins (Massague, 1998).

6.1.2 TGFβ1 regulation of megakaryocytopoiesis

Regulation of haemopoiesis by TGFβ1 is complex, and TGFβ1 can have a positive or negative effect on proliferation and maturation depending on the lineage and/or developmental stage of the target cell. However, in the MK lineage, TGFβ1 acts as an inhibitor of both proliferation and maturation.

Proliferation assays using human BM CD34+ cells cultured in human plasma demonstrated that the percentage of MK cells produced was reduced 3-4 fold compared to serum free cultures supplemented with a standard cytokine cocktail (IL-3, IL-6 and IL-11). Addition of a neutralising TGFβ1 monoclonal antibody to the cultures containing plasma restored MK growth to the level of the serum free cultures, indicating that TGFβ1 was the factor inhibiting proliferation of the MK lineage (Berthier *et al*, 1993). Formation of HPP-CFU-MK in cultures of murine BM cells was completely abrogated in the presence of TGFβ1 (Jackson *et al*, 1994b). Also, murine BM cells grown in liquid culture in the presence of TGFβ1 showed a 50% decrease in AChE activity, a marker of MK maturity, and only 20% of single MKs in culture showed a size increase compared to 68% without TGFβ1 demonstrating inhibition of MK maturation (Ishibashi *et al*, 1987).

TGFβ1 inhibition of the MK lineage may occur, at least in part, through the IL-3 and/or TPO signalling pathways. IL-3 promoted MK colony formation was inhibited by 10-200 pg/ml TGFβ1 in cultures of murine BM progenitor cells. However, inhibition could not be demonstrated in cultures stimulated by IL-6, even using 200-500 pg/ml TGFβ1, implying specific suppression of IL-3 stimulation (Han *et al*, 1992). Inhibition of IL-3 induced MK formation occurs at the CFU-MK stage of differentiation as CFU-MK expansion stimulated by IL-3 is inhibited by TGFβ1, but not CFU-MK expansion stimulated by IL-1 or IL-6 (Jackson *et al*, 1994b). Formation of the less mature colony forming unit HPP-CFU-MK is inhibited by TGFβ1 whether stimulated by IL-1, IL-3 or IL-6.

As well as the specific inhibition of IL-3 mediated MK proliferation and differentiation, TGFβ1 inhibits MK growth mediated by TPO. Cultures of the TPO responsive cell line Mo-7e containing 100ng/ml of either TPO, TGFβ1 or both were analysed for proliferation by ^{3}H thymidine incorporation. Cultures stimulated with TPO showed a dose-dependent 6 fold increase in thymidine incorporation compared to unstimulated control, but thymidine incorporation in cultures containing TGFβ1 were not significantly different to unstimulated control. When both TGFβ1 and TPO were added to cultures of Mo-7e cells, the growth enhancing effect of TPO was completely abrogated and no significant difference in thymidine incorporation could be demonstrated compared to unstimulated control cultures (Kalina *et al*, 2001). Inhibition of TPO stimulated megakaryocytopoiesis may occur by modulation of TPO expression as human BM stromal cells cultured with TGFβ1 have been shown to decrease significantly the expression of TPO mRNA in a dose-dependent fashion (Sungaran *et al*, 2000). However, TPO expression can also be up-regulated by low dose TGFβ1 in the BM microenvironment as discussed more fully in Chapter 5 (5.1.4)

Platelets from gray platelet syndrome patients, a condition where platelets have little or no granules, showed only minor inhibition of MK growth in human BM cultures, further suggesting that TGFβ1 inhibits MK growth (Mitjavila *et al*, 1988).

6.1.3 TGFβ1 in cMPDs

TGFβ1 levels may be dysregulated in cMPD. The mean level of (a+l) TGFβ1 from 10 healthy controls was 320 ±52 ng/10^9 platelets, but was raised to between 420-900 ng/10^9 platelets in 7 patients with cIMF ($p<0.003$) (Martyre *et al*, 1991). This raised TGFβ1 level was confirmed in platelet lysates from 4 cIMF patients by a second group (Zauli *et al*, 1993). Expression of TGFβ1 mRNA was elevated in PBMNC of 7 out of 8 patients with cIMF compared to healthy controls by northern blot analysis (Martyre *et al*, 1994). An increase in secreted peptide was also identified, and immunohistochemistry demonstrated co-localisation of antibodies to TGFβ1 and $gpII_bIII_a$ indicating localisation of the TGFβ1 to MKs. However, BM biopsies from 11 cIMF patients showed no difference in MK staining intensity using an antibody to TGFβ1 (Chou *et al*, 2003). Also, no difference in TGFβ1 expression levels could be detected in BM CD34+ cells from 8 cIMF patients by RT-PCR analysis (Bousse-Kerdiles *et al*, 1996). This suggests that TGFβ1 regulation between the

BM microenvironment and circulating platelets may be disrupted, and this may be important in the pathogenesis of cIMF.

Four groups have reported TGFβ1 levels in ET. In one study, levels of (a+l) TGFβ1 in platelet lysates from 7 ET patients were similar to normal controls (Zauli *et al*, 1993). However, other studies have shown that circulating TGFβ1 levels in ET patients were raised in comparison to normal controls.

TGFβ1 levels were measured from serum samples obtained from 16 normal controls, 10 clonal ET patients and 12 polyclonal ET patients. The level of TGFβ1 in normal controls was 34.4ng/ml (range 22.8-56.0), 44.4ng/ml in clonal ET patients (range 23-108.1) and 46.4ng/ml (range 4.8-71) in polyclonal ET patients. While there was no significant difference between the three cohorts, and the range of results was large and overlapping, there was a trend towards higher serum TGFβ1 levels in ET (Harrison *et al*, 1999b). Because *in vitro* release of TGFβ1 from platelets may affect results from serum samples, plasma TGFβ1 levels were also measured. Plasma TGFβ1 levels for normal controls were 5.1ng/ml (range 1.6-5.8), 8ng/ml (range 4.3-11.4) for 5 clonal ET patients, 14ng/ml (range 4.4-22.6) for 5 polyclonal ET patients and 5.6ng/ml (range 5.6-11.9) for 5 RT patients. The plasma data showed a more pronounced increase in TGFβ1 levels in ET patients compared to controls, although a significant difference was still not reached, probably due to small patient numbers (Harrison *et al*, 1999b). In a second study mean plasma levels of both active (371.14 ±205.35 pg/ml) and latent (17.07 ±7.13 ng/ml) TGFβ1 in 10 ET patients were shown to be significantly higher than those in 6 healthy volunteers (95.50 ±4.95 pg/ml and 6.57 ±2.68 ng/ml respectively) (Kuroda *et al*, 2004). A third study also showed that plasma TGFβ1 levels of 1,405 pg/ml (range 852-1,851.4) in 14 ET patients were significantly higher ($p<0.0001$) than 606.9pg/ml (range 286.5-858) for controls (Lev *et al*, 2002). However, no significant difference in TGFβ1 levels from platelet lysates could be demonstrated between ET patients and normal controls in this third study.

When taken together these studies suggest that levels of TGFβ1 in platelets from ET patients may be normal, but circulating levels appear to be increased. While this appears counter intuitive in ET patients, since increased levels of an inhibitor of megakaryocytopoiesis would be expected to lead to a decrease in MK proliferation, ET patients may be resistant to the inhibitory effects of TGFβ1.

In 8 ET patients, CFU-MK derived from BM CD34+ cells were significantly ($p<0.05$) less responsive to the inhibitory activity of platelet lysates than CFU-MK derived

from BM CD34+ cells from 8 normal controls. A 10 fold higher platelet lysate concentration was required to inhibit CFU-MK derived from ET BM CD34+ cells than that needed for CFU-MK derived from normal BM CD34+ cells (Zauli *et al*, 1993). Reversal of all inhibition occurred by addition of anti-TGFβ1 antibodies, suggesting that TGFβ1 was the main factor responsible for the inhibition.

This data has been confirmed in a second cohort of 10 ET patients. The percentage suppression of 1ng/ml TGFβ1 on CFU-MK derived from BM CD34+ cells from 5 normal controls was 95 ±2.6%, but this was reduced to 56 ±11.8% for CFU-MK derived from BM CD34+ cells from ET patients (Kuroda *et al*, 2004). This reduced inhibition was associated with low level expression of Smad4, a down stream effector molecule in TGFβ1 signalling, in 5 patients studied from the ET cohort. Transfection of the Smad4 gene into BM MNC from ET patients restored the inhibitory effects of TGFβ1. Thus ET MK progenitors may not be inhibited by TGFβ1 to the same extent as normal MK progenitors, and this may be due to decreased expression of Smad4.

6.1.4 TGFβ1 Polymorphisms

A number of polymorphisms have been described at the TGFβ1 gene locus. Using RT-PCR-SSCP a single group described 5 SNPs in TGFβ1 (Awad *et al*, 1998). Four of these polymorphisms occur either in the promoter or coding regions of the gene and may affect TGFβ1 expression. A G to A and a C to T substitution occur 800bp and 509bp upstream of the AUG respectively, in the promoter region of the gene. The other two polymorphisms, a T to C substitution at nucleotide 869 and a G to C substitution at nucleotide 915, occur within the signal peptide. A number of studies investigating the effects of these polymorphisms have demonstrated their involvement in disease.

The polymorphisms in the promoter region of TGFβ1 were both identified in lung carcinoma patients, but were not thought to be linked to disease (Awad *et al*, 1998). However, in 170 pairs of twins a T allele at position -509 of the TGFβ1 gene led to an increase in serum concentration of (a+l) TGFβ1 as measured by ELISA. The mean TGFβ1 level in CC homozygous subjects was 3.83 ng/ml, in TC heterozygous individuals 5.06ng/ml, and in homozygous TT individuals 7.62 ng/ml, suggesting that TGFβ1 expression may be influenced by the genotype at the TGFβ1 locus (Grainger *et al*, 1999).

At codon 10 a T869C polymorphism, which changes a leucine to a proline, is linked to osteoporosis (Yamada *et al*, 1999). Possessing the C allele (the higher producer allele) was shown to confer resistance against osteoporosis. In a study of 356 healthy Japanese students a 5-6% increase in bone mineral density was measured in subjects homozygous for the C allele compared to those homozygous for T alleles, with a 2% increase for heterozygous individuals. This was thought to occur due to a local increase of TGFβ1 in the bone marrow leading to higher proliferation of osteoclasts for which TGFβ1 is a promoter of proliferation.

The G915C substitution in codon 25 converts an arginine, a large charged polar amino acid, to a proline, a small nonpolar amino acid, at codon 25 of the preproTGFβ1 translation product and is associated with inter individual variation in plasma TGFβ1 concentration. This polymorphism falls in the signal sequence of the protein, and may alter the efficiency with which TGFβ1 processing can occur, thus changing its rate of release. Stimulated lymphocytes cultured from homozygous G individuals produced 10,037 ± 745 pg/ml TGFβ1 compared to 6,729 ± 883 pg/ml ($p<0.02$) for heterozygous individuals. This has also been linked to disease pathology. Lung transplant patients with the G allele demonstrate higher levels of fibrosis post transplant than other patients (Awad *et al*, 1998).

A shift to the higher producer allele at any of these polymorphic loci could lead to the increase in the circulating levels of TGFβ1 described in ET patients.

6.1.5 IL-6

Originally described as B-cell stimulating factor because it induces maturation in B-cells, IL-6 is the founder member of the IL-6 cytokine family (Hirano *et al*, 1986). A 26 KDa protein, IL-6 consists of 212 amino acids, the first 28 being the signal peptide. Its specific interaction with the IL-6 receptor (IL-6R) leads to signal transduction via gp130 dimerisation and tyrosine phosphorylation (Hibi *et al*, 1996; Hirano *et al*, 1997).

IL-6 has been shown to stimulate platelet production. IL-6 administered to mice led to a dose-dependent increase in platelet count. A 50-60% increase in platelet number was seen at plateau, associated with an increase in megakaryocyte size, but not number (Ishibashi *et al*, 1989a). This size increase was associated with an increase in average ploidy from 16N to 32N, indicating that IL-6 is important for MK differentiation but does not appear to affect proliferation (Ishibashi *et al*, 1989a; Hill *et al*, 1991). IL-6 alone did not

influence CFU-MK formation from murine BM cells, but did augment the number of colonies produced when IL-3 was added (Ishibashi *et al*, 1989b). Also, cultures of murine BM cells treated with IL-6, G-CSF, GM-CSF and/or IL-3 in various combinations demonstrated that other cytokines augment the action of IL-6 in either a synergistic or additive fashion (Quesenberry *et al*, 1991). Therefore, IL-6 can stimulate proliferation as well as maturation, but only in the presence of other factors.

Studies using human BM or MK cells have similarly shown that IL-6 alone can stimulate the maturation of MKs and works in synergy or addition with other growth factors on proliferation. No increase in CFU-MK was demonstrated in cultured human BM or cord blood cells stimulated by IL-6 alone, but an increase in size and DNA content up to 16N was recorded (Imai *et al*, 1991; Kimura *et al*, 1990). However, by introducing other cytokines into culture mixtures, IL-6 did have an effect on human MK proliferation. The addition of IL-6 to cultures of human MKs containing IL-3 led to a 2 fold increase of the proliferation seen with IL-3 alone (Bruno & Hoffman, 1989). Also, human BM cells enriched for MKs gave no colony growth with IL-6 or IL-1α alone, but cultures containing both cytokines did proliferate (Bruno *et al*, 1991b). Thus IL-6 is able to stimulate proliferation and maturation of human MKs *in vitro*. In fact the combination of SCF, IL-3 and IL-6 has been shown to be as potent as TPO for MK growth in cultures of CD34+ cells, although the number of platelets produced was 10 fold lower than with TPO alone, suggesting that IL-6 can stimulate an increase in number, size and DNA content in MK, but is not involved in platelet shedding (Bruno *et al*, 1991b; Norol *et al*, 1998).

Administration of IL-6 to patients with chemotherapy-induced thrombocytopenia led to significant increases in platelet counts *in vivo*. However, it is not in common use as a therapeutic agent due to a high level of systemic toxicity (D'Hondt *et al*, 1995; Gordon *et al*, 1995; Schrezenmeier *et al*, 1995; van Gameren *et al*, 1994).

Platelet counts in the IL-6 knockout mouse were within the normal range at approximately $900x10^6$/ml and mice developed normally, suggesting that while IL-6 can augment platelet production, it is not essential for normal thrombopoiesis (Gainsford *et al*, 2000; Kopf *et al*, 1994). In mpl knockout mice, which have platelet counts of around $100x10^6$/ml, the platelet production is not due to the action of IL-6, as mpl/IL-6 double knockouts have comparable platelet counts (Gainsford *et al*, 2000). Thus, while increasing availability of IL-6 *in vivo* can increase platelet counts, reducing IL-6 levels does not lead

to decreased platelet counts, suggesting that normal thrombopoiesis is not reliant on stimulation from IL-6.

IL-6 may affect thrombopoiesis *in vivo* via stimulation of TPO expression. As the *in vitro* experiments demonstrated no increase in platelet numbers with the addition of IL-6, but *in vivo* administration in mice and humans has resulted in platelet count increases, it is clear that other factors are involved in augmentation of platelet numbers by IL-6. Upon administration of 30 μg/kg/d IL-6 to cancer patients, plasma TPO levels increased from basal levels of 152 ±35 pg/ml to 630 ±110 pg/ml. Platelet counts in normal mice increased with IL-6 administration from 1,025 ±80 $x10^6$/L to 1,492 ±70 $x10^6$/L, and this increase was accompanied by TPO plasma level increases of >2.5 fold (Kaser *et al*, 2001). Neutralising antibodies to TPO severely abrogated the thrombopoietic effect of IL-6, suggesting that the increase in platelet counts caused by IL-6 administration was due to increased TPO signalling.

A number of groups have measured IL-6 levels in ET patients with conflicting results. Serum IL-6 levels in samples from 13 ET patients were 1.56 U/ml ±1.2, which was not significantly different from levels measured in 71 control samples, 2.19 U/ml ±1.08 (Hollen *et al*, 1991; Hollen *et al*, 1992). Similar results have been reported in plasma samples where a mean value of 0.33 ±0.12 fmol/ml was recorded in 45 samples from ET patients and 0.31 ±0.19 in 25 normal control samples (Alexandrakis *et al*, 2003). Median IL-6 levels in plasma samples from 10 clonal ET patients were 4 pg/ml (range 0-14) which was not significantly different from 12 polyclonal patients, median 3.8 (range 0-12) or 16 normal controls, median 6 (range 0-19) suggesting that the levels of IL-6 in plasma from ET patients is not related to clonality status (Harrison *et al*, 1999b).

However, elevated IL-6 levels have been reported in a cohort of 37 patients with cMPD containing 16 ET, 10 cIMF, 8 CML and 3 PV patients. Mean IL-6 levels in serum samples from the 37 patients with cMPD were 1.6 ±0.4 pg/ml, significantly raised in comparison to 0.2 ±0.1 pg/ml for normal controls ($p<0.02$) (Uppenkamp *et al*, 1998). Only 8 of the 37 cMPD patients had IL-6 levels above the normal range, and the diagnosis of these 8 patients is not documented, however, this result indicates that elevated IL-6 levels may be involved in the pathogenesis of ET in at least a small number of cases.

6.1.6 IL-6 polymorphisms

Since administration of exogenous IL-6 can lead to an increase in platelet count, a polymorphism resulting in an intrinsic increase in circulating IL-6 levels may have a similar effect and could contribute to the pathogenesis of ET. A single nucleotide polymorphism, a G to C at nucleotide -174 in the promoter region of the IL-6 gene has been identified which affects plasma levels of IL-6. A study of fasting plasma IL-6 levels in samples from 102 healthy Caucasians measured median IL-6 levels for individuals homozygous GG as 2.74 pg/ml (95% CI 2.43-3.10), heterozygous GC 2.64 pg/ml (95% CI 2.35-2.97) and homozygous CC 1.63 (95% CI 1.44-1.86) showing that G at position -174 is the higher producer allele (Fishman *et al*, 1998).

The G-174C polymorphism is a risk factor in a number of disorders. Following BM transplantation, patients can develop a systemic toxicity to the donor cells termed graft verses host disease (GVHD) which is caused by repopulating donor immune cells reacting to host antigen. Circulating IL-6 levels are elevated in both chronic (cGVHD) and acute GVHD (aGVHD) and in aGVHD, IL-6 levels correlate with severity and prognosis. In a study of 80 patients BM transplanted for haematological malignancy, patients with a G allele at nucleotide -174 had a tendency to higher grades of aGVHD and patients homozygous for the G allele had an increased incidence of chronic GVHD compared to patients homozygous for the C allele (Cavet *et al*, 2001). This suggests that the G allele can directly influence disease.

A number of HIV patients suffer with Kaposi sarcoma (K-S), an angioproliferative disorder associated with lesions containing high levels of IL-6, and which may progress to malignancy. In a cohort of 115 HIV patients, homozygotes for the G allele were over represented in the K-S group, and homozygous C patients under represented, compared to 125 HIV patients without K-S (Foster *et al*, 2000), suggesting that carrying the G allele may be a risk factor for K-S.

A disorder associated with changes in IL-6 levels is systemic juvenile chronic arthritis (sJCA). One of the main symptoms of sJCA is a spiking fever which correlates with spikes in IL-6 serum levels. In a study of 97 sJCA patients less severe symptoms occurred in those with the CC genotype compared to other genotypes (Fishman *et al*, 1998), suggesting that a G allele at nucleotide -174 is an important modulator of disease severity for sJCA.

6.1.7 Aim

The aim of the work detailed in this chapter was to analyse the four polymorphisms previously reported in the TGFβ1 gene, and the G-174C polymorphism described in the IL-6 gene, in a cohort of ET patients and haematologically normal controls, using PCR and restriction enzyme analysis.

6.2 Materials and Methods

6.2.1 Patients and Samples

Peripheral blood samples were collected from 75 patients with a sustained thrombocytosis, 66 with a diagnosis of ET according to the PVSG criteria and 9 with a provisional diagnosis of ET but with the PVSG criteria yet to be confirmed, and 100 haematologically normal controls. Of the 75 patients, 11 were clonal and 24 were polyclonal by XCIP analysis, the other 40 samples were uninterpretable due to age or a constitutively imbalanced XCIP, or were male. DNA was prepared from purified neutrophils as described in chapter 2 (2.2.1, 2.2.2).

6.2.2 PCR

Approximately 100ng of DNA was used for 35 cycles of PCR using the primers listed in Table 6.1, at an annealing temperature of 64°C. The location of the PCR primers and the polymorphisms in the TGFβ1 and IL-6 genes are shown in Figure 6.2.

6.2.3 Analysis of polymorphisms

PCR was carried out as described above using neutrophil DNA. All TGFβ1 restriction digests contained 3μl of PCR product with BSA and appropriate buffer in a final volume of 6μl. The polymorphisms at -800 and -509 were investigated using the product of primers TB1/F and TB1/R, the G-800C polymorphism was analysed using *BsaH*I, and the C-509T polymorphism with *Afl*II. Polymorphisms at 869 and 915 were investigated with primers TB2/F and TB2/R, the T869C polymorphism was analysed using *Msp*AI, and the G915C polymorphism using Sau96 I. The IL-6 polymorphism at nucleotide -174 was analysed using primers IL-6/F and IL-6/R with *Hinf*I digestion. The *Hinf*I digestion

Primer Name	Primer Sequence 5'→3'
TB1/F	CGTGGAGTGCTGAGGGACTCTGCCTCCGAC
TB1/R	AGTGGGAGGAGGGGGCAACAGGACACCTTA
TB2/F	CCACCCACCTTCTGGTACCAG
TB2/R	TCTTGCAGGTGGATAGTCCCG
IL-6F	GGCTGCGATGGAGTCAGAGGA
IL-6R	AAGATTGTGCAATGTGACGTCCTTTAGAAT

Table 6.1
Table of Primers used to study the TGFβ1 and IL-6 polymorphisms
Primer names and sequences, with mismatched bases underlined.

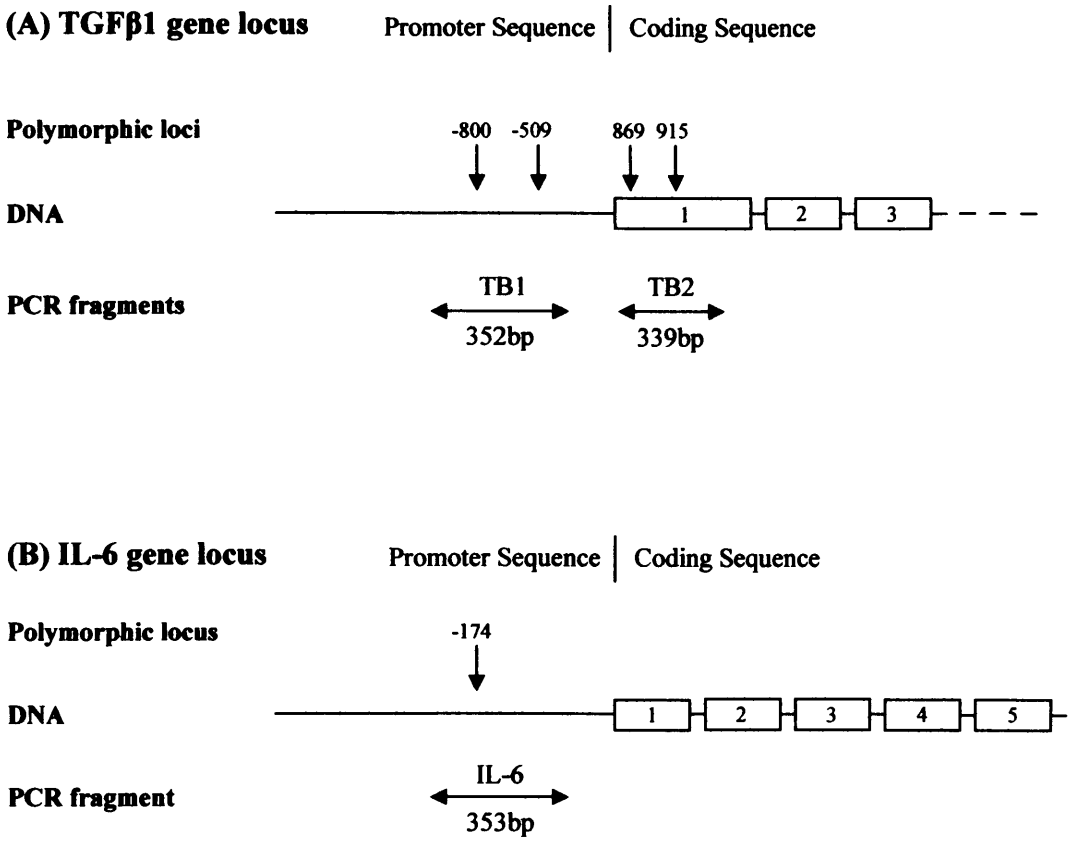

Figure 6.2
Gene structure of the TGFβ1 and IL-6 loci

Location of the (A) TGFβ1 and (B) IL-6 polymorphisms and the PCR fragments used to analyse them. The boxes represent exons and the solid lines represent introns

contained 10µl of PCR product and 1x buffer 2 in a final volume of 12µl without BSA. All PCR products were digested overnight at 37°C according to manufacturers instructions (NEB). The samples were then electrophoresed through agarose gels and visualized by ethidium bromide staining. Sizes of fragments expected from the digestions are shown in Figure 6.3 and Figure 6.4 shows the digestion patterns for each genotype visualised by ethidium bromide staining of agarose gels.

6.2.4 Confirmation of Digests

Inconsistencies in the intensity of the 108bp band were observed in the digestion patterns obtained during analysis of the polymorphism in the IL-6 gene. Therefore, a small number of samples were sequenced to confirm the results. A 50µl PCR using the IL-6 primers was prepared, cleaned using Wizard PCR clean-up kit (Promega) and eluted into 50µl of distilled water. Cycle sequencing was then carried out as described in chapter 2 (2.2.9) using 20ng of PCR product, the IL-6/F primer and BigDye Terminator Ready Reaction Mix (Applied Biosystems).

6.2.5 Statistics

χ^2 analysis was carried out using Microsoft Excel XP.

6.3 Results

6.3.1 Polymorphism analysis of TGFβ1

The G to C polymorphism at nucleotide -800 of the TGFβ1 gene was analysed in 75 ET patients and 100 normal controls. The ET patient cohort had a gene frequency for G of 0.93, normal controls had a gene frequency for G of 0.94 (Figure 6.5A). The percentages of the three genotypes in the ET patients were 87% homozygous GG, 12 percent were heterozygous GC and 1% were homozygous for the CC allele. The percentages of each genotype were similar in normal control samples, 87% were homozygous GG, 13% were heterozygous GC and none were homozygous for CC alleles. No difference in genotype or gene frequency could be demonstrated between clonal and polyclonal patients either. The gene frequency for G in 11 clonal patients was 1.0 and in 24 polyclonal patients was 0.9.

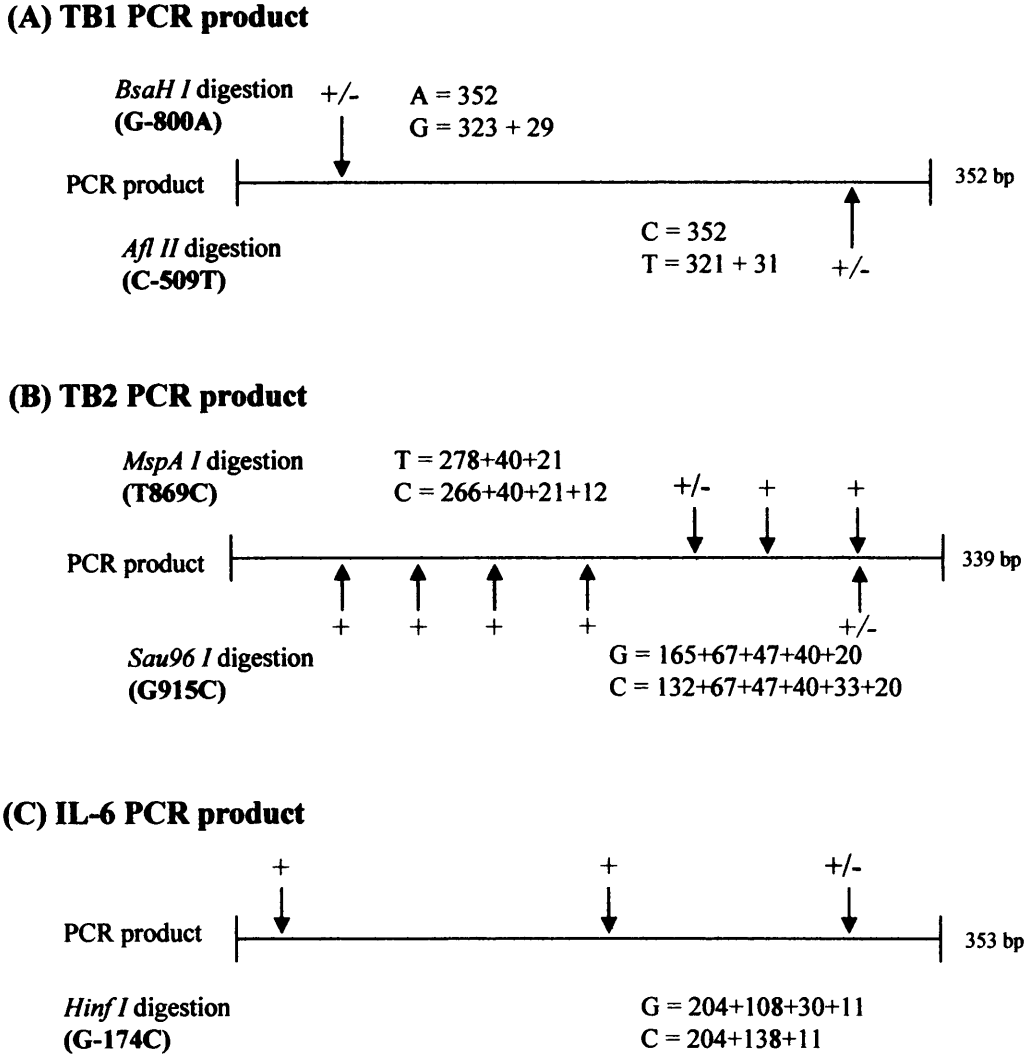

Figure 6.3
Restriction enzyme digests for the TGFβ1 and IL-6 polymorphisms.

Diagram showing the three PCR fragment sizes, enzyme cutting sites and digested product sizes for each of the five polymorphisms

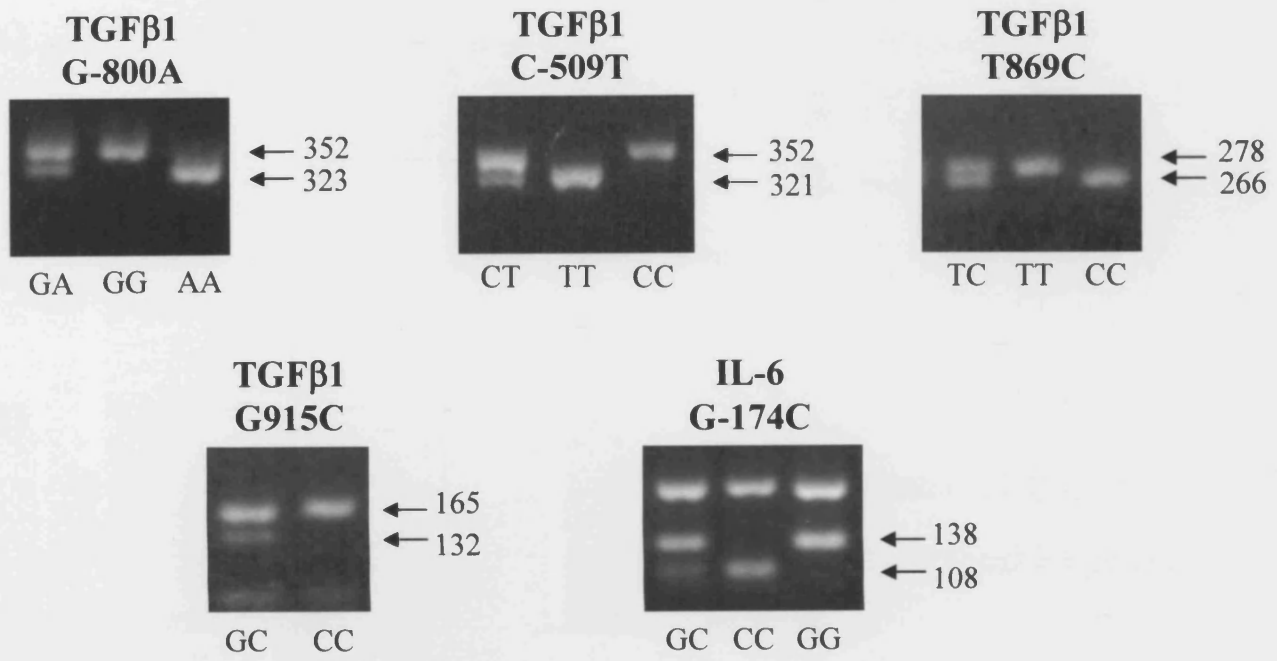

Figure 6.4
Restriction enzyme digest patterns for the TGFβ1 and IL-6 polymorphisms

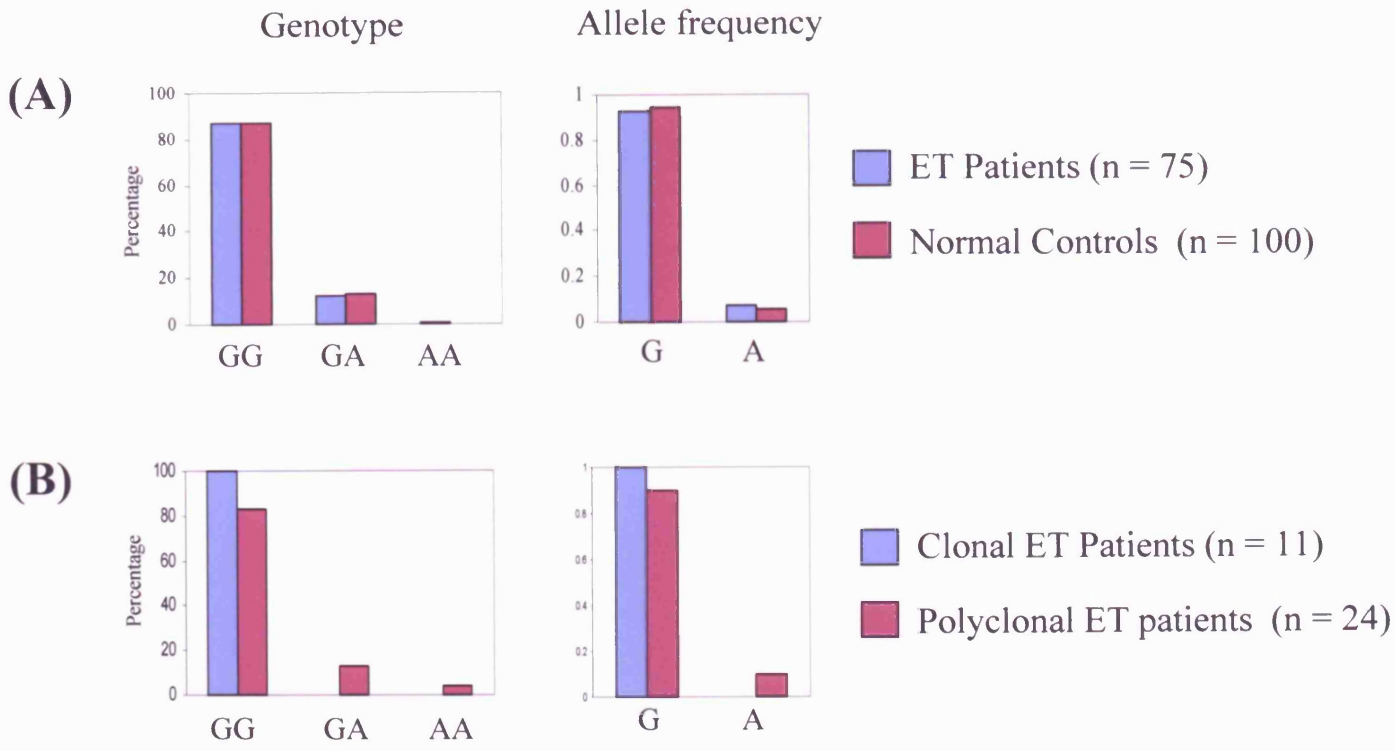

Figure 6.5
The genotypes and allele frequencies for the G-800A TGFβ1 promoter polymorphism in :-
(A) All ET patients and normal controls
(B) Clonal and polyclonal ET patients.

All clonal patients were homozygous GG, polyclonal patients were 83% GG, 13% heterozygous GA and 4% homozygous AA (Figure 6.5B).

At nucleotide -509 a C to T polymorphism was analysed in 75 ET patients and 100 normal controls. The gene frequency of the CC allele was 0.71 for ET patients, and 0.70 for normal controls (Figure 6.6A). The percentage of patients with each genotype was 52% for homozygous CC patients, 39% for heterozygous CT patients and 9% for patients homozygous for the TT allele. The percentages of each genotype in normal control subjects were homozygous CC 48%, heterozygous CT 44% and homozygous TT 8%. No significant difference in genotype or gene frequency could be demonstrated between clonal and polyclonal patients either. The gene frequency for C in 11 clonal patients was 0.78 and in 24 polyclonal patients was 0.65. Clonal patients were homozygous CC 64%, heterozygous CT 27% and homozygous TT 2%. Polyclonal patients were 38% CC, 54% heterozygous CT and 8% homozygous TT (Figure 6.6B). While clonal and polyclonal patient genotypes were slightly different, this is most likely due to the small size of each patient cohort rather than disease specificity, although investigation of this polymorphism in a larger cohort would be useful.

These data suggest that there are no significant differences in allele frequency or genotype between ET patients and normal controls, or between clonal and polyclonal ET patients, at either of the polymorphic loci in the promoter region of TGFβ1 gene.

The T to C polymorphism at nucleotide 869 of the TGFβ1 gene was analysed in 67 ET patients and 100 normal control subjects. The allele frequency of T alleles in ET patients was 0.68 and in normal controls was 0.62 (Figure 6.7A). The percentages of the three genotypes in the ET patients were 45% homozygous T, 46% heterozygous TC and 9% homozygous C. The percentages of each genotype were similar in normal control samples, 36% were homozygous T, 51% were heterozygous TC and 13% were homozygous for C alleles. The gene frequency for C in 11 clonal patients was 0.37 and in 24 polyclonal patients was 0.3. 9% of clonal patients were homozygous CC, 55% heterozygous CT and 36% homozygous TT. Polyclonal patients were 4% CC, 52% heterozygous CT and 43% homozygous TT (Figure 6.7B).

At nucleotide 915 the G to C polymorphism was analysed in 75 ET patients and 100 normal controls. The allele frequency for the G allele was 0.93 for ET patients and 0.94 for normal controls. The percentages of each genotype in ET patients were GG 85%, CG 15% and CC 0%, and the percentages in normal control subjects were very similar, GG 88%, GC

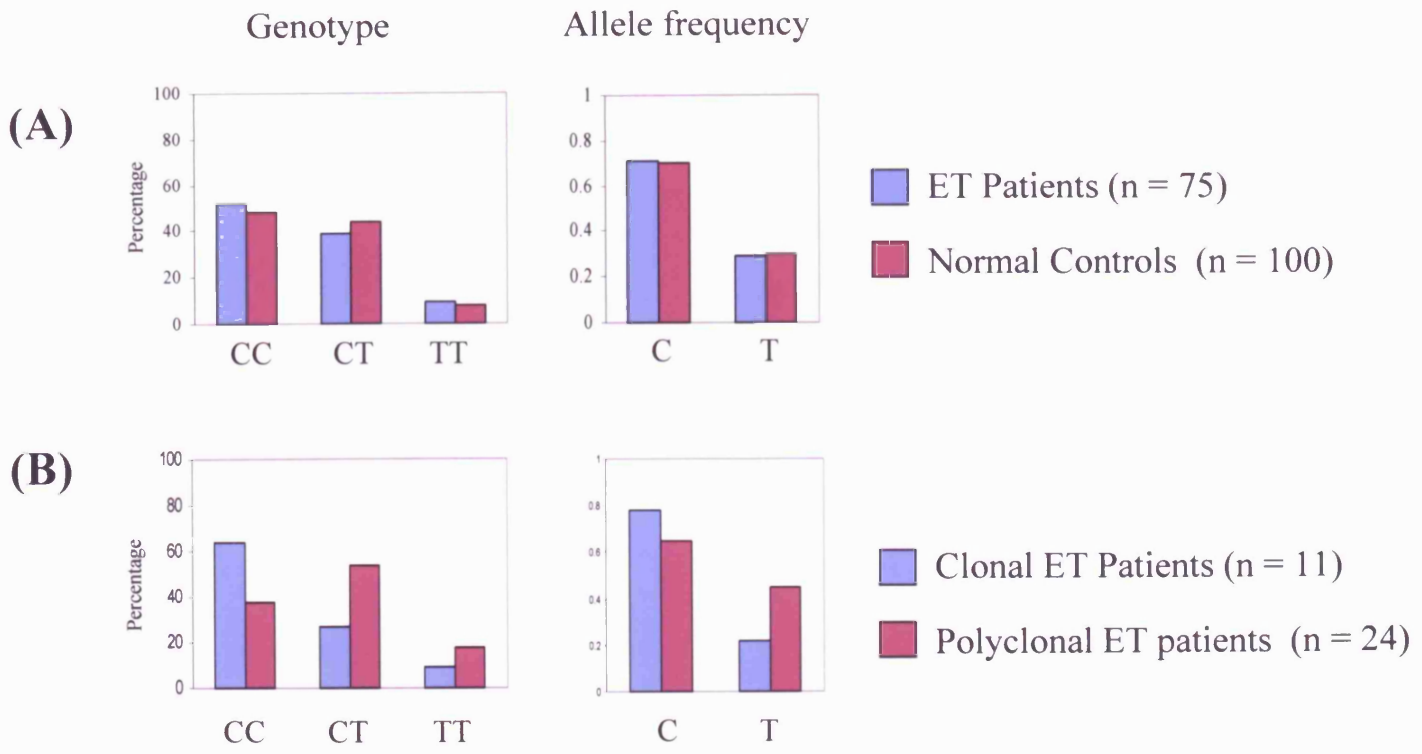

Figure 6.6
The genotypes and allele frequencies for the C-509T TGFβ1 promoter polymorphism in :-
(A) All ET patients and normal controls
(B) Clonal and polyclonal ET patients.

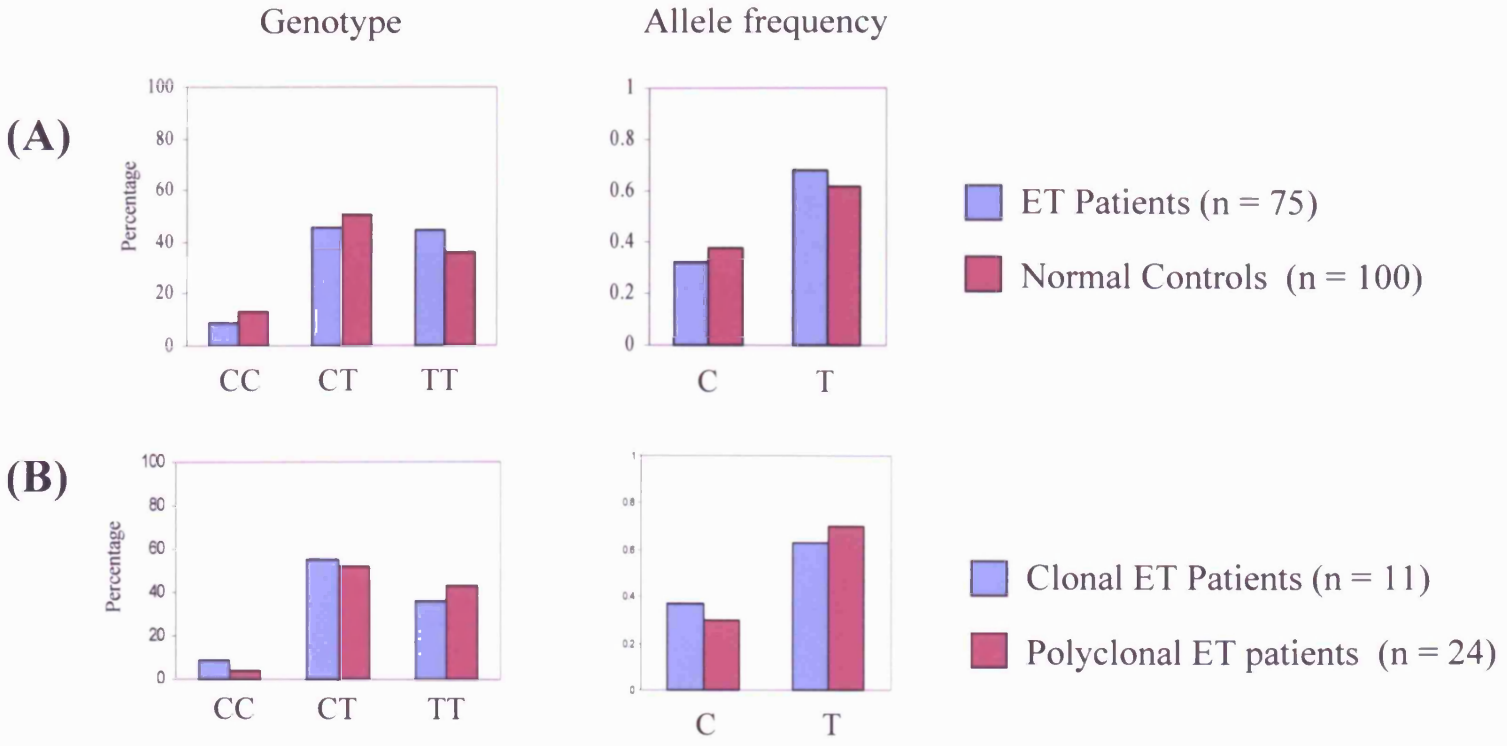

Figure 6.7
The genotypes and allele frequencies for the C869T TGFβ1 coding sequence polymorphism in :-
(A) All ET patients and normal controls
(B) Clonal and polyclonal ET patients.

12% and CC 0% (Figure 6.8A). Clonal patients had a gene frequency of 0.86 for G alleles and polyclonal patients 0.96. The percentages of each genotype at 915 of the TGFβ1 gene in clonal patients were 0% homozygous CC, 27% heterozygous CG and 73% homozygous GG. Polyclonal patients were not significantly different with 0% homozygous CC, 8% heterozygous CG and 92% homozygous GG (Figure 6.8B).

Thus no significant differences in allele frequency or genotype between ET patients and normal controls could be demonstrated at either of the polymorphic loci in the coding region of TGFβ1 gene.

6.3.2 Polymorphism analysis of IL-6

The G-174C polymorphism in the promoter region of the IL-6 gene was analysed in 75 ET patients and 100 normal controls. The allele frequency in ET patients for the G allele was 0.65 and in normal controls was 0.61 (Figure 6.9A). The percentages for each genotype in ET patients were 39% homozygous GG, 52% heterozygous GC and 9% homozygous CC. In normal controls the percentages were 45% homozygous GG, 33% heterozygous GC and 22% of controls were homozygous for the C allele. There are minor differences in the percentages of each genotype between ET patients and normal controls. However, the gene frequency of each allele is the same in ET patients as normal controls and the sample size is small. 55% of clonal patients were heterozygous CG and 45% were homozygous GG, polyclonal patients were 50% CG and 50% GG. No CC homozygotes were present in either clonal or polyclonal cohorts (Figure 6.9B). As there is no difference between the results for clonal and polyclonal patients or normal controls it is unlikely that the G-174C polymorphism in the promoter region of the IL-6 gene is important for the pathogenesis of ET.

6.3.3 Sequencing of the IL-6 gene

Analysis of the IL-6 polymorphism was complicated by problems with the digest pattern. Alleles with a G at nucleotide -174 would produce bands at 204bp, 108bp, 30bp and 11bp upon digestion of the IL-6 PCR product with *HinfI*, and digestion of C containing alleles would produce bands of 204bp, 138bp and 11bp. The heterozygote could be difficult to determine due to variation in the intensity of the 108bp band. To test the accuracy of the digest results, four samples were prepared for sequencing. Two heterozygous individuals were investigated, one with a high intensity band at 108bp and another with a low intensity

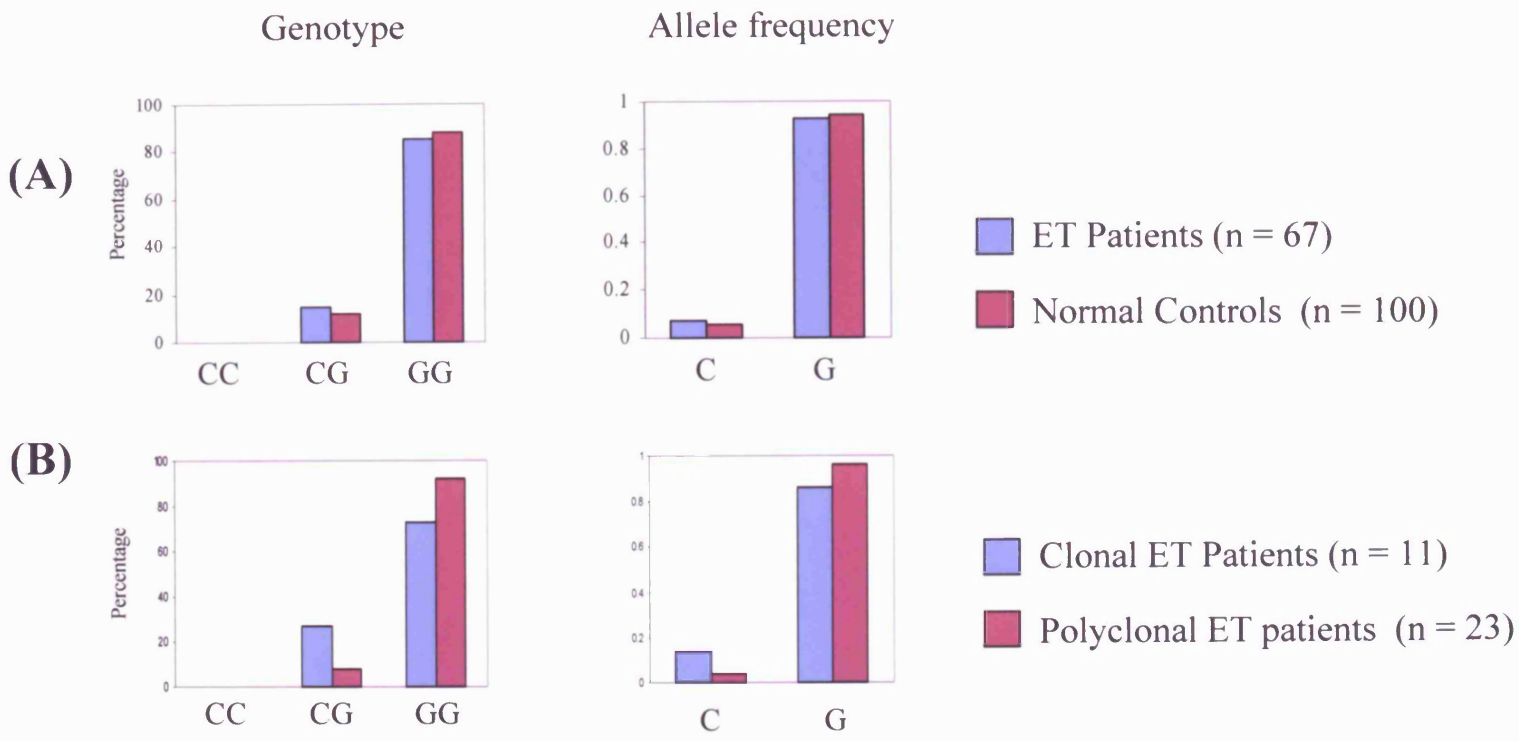

Figure 6.8
The genotypes and allele frequencies for the C915G TGFβ1 coding sequence polymorphism in :-
(A) All ET patients and normal controls
(B) Clonal and polyclonal ET patients.

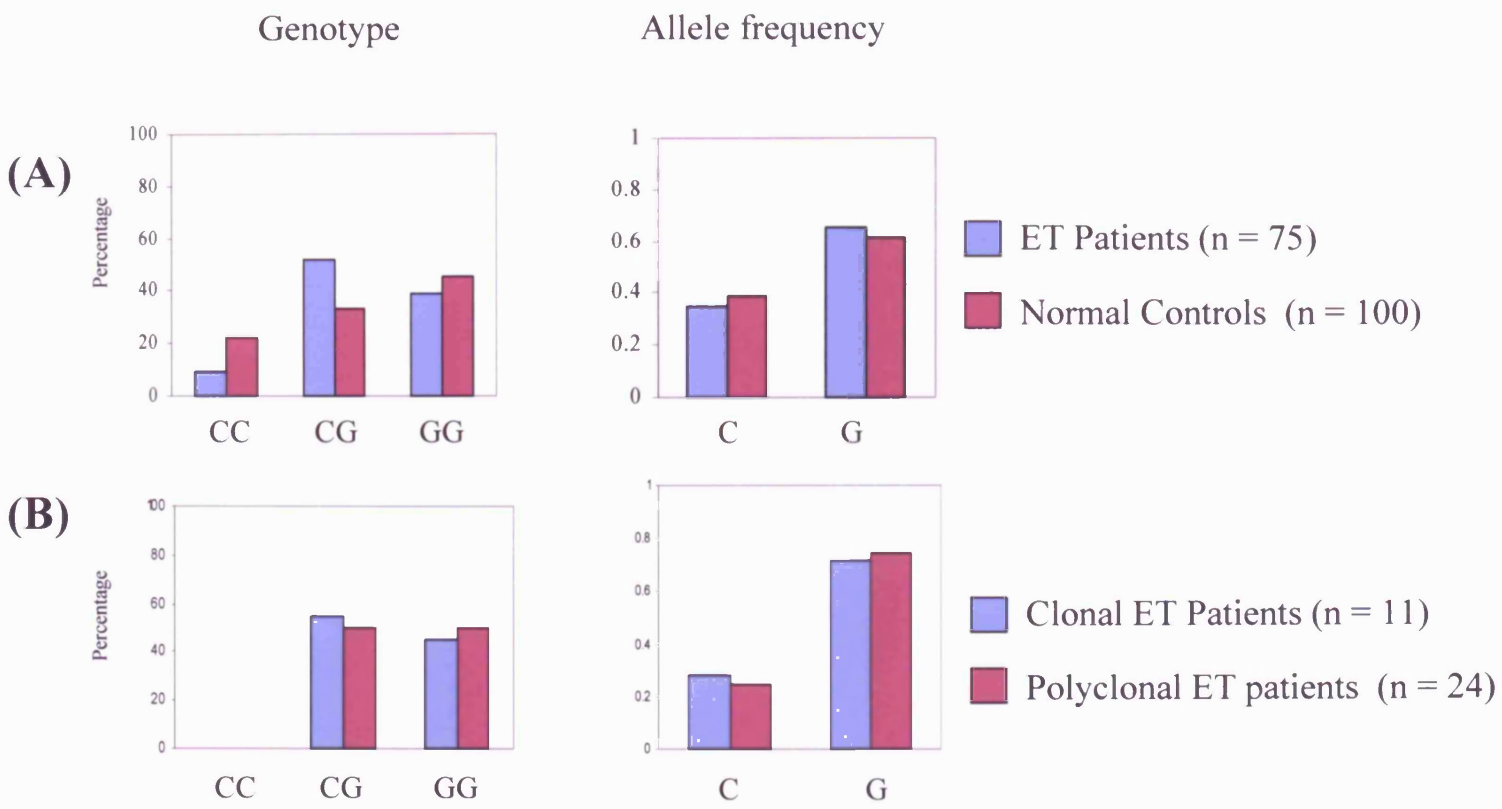

Figure 6.9
The genotypes and allele frequencies for the C-174G IL-6 promoter polymorphisms in :-
(A) All ET patients and normal controls
(B) Clonal and polyclonal ET patients.

band at 108bp. Two other individuals were sequenced, a GG homozygote and a CC homozygote, to confirm the results (Figure 6.10). Both the individuals heterozygous by restriction enzyme analysis were shown to have both C and G alleles. The sample with a more intense band at 108bp (Figure 6.10A) also had a larger G peak at nucleotide -174 compared to the sample with a less intense band at 108bp (Figure 6.10B). However, this is likely to be technical variation relating to the sequencing analysis. The size of the G peak at nucleotide -173 is also significantly different between the two samples and it is likely that this is affecting the height of the G peaks at nucleotide -174.

6.4 Discussion

TGFβ1 and IL-6 are able to alter platelet production *in vitro* and *in vivo*. TGFβ1 is a negative regulator of thrombopoiesis, and IL-6 is a positive regulator of thrombopoiesis. Polymorphisms have been identified in the TGFβ1 and IL-6 genes which can affect protein expression. Two polymorphisms are in the promoter region of the TGFβ1 gene, two are in the signal peptide encoding region of the TGFβ1 gene and one polymorphism is in the promoter of the IL-6 gene. The work described in this chapter has investigated both the allele frequency and genotype at each of these five polymorphic loci to establish whether they might have a role in the pathogenesis of ET. All polymorphisms were analysed using PCR and restriction enzyme digestion of DNA extracted from neutrophils obtained from a cohort of ET patients and 100 haematologically normal individuals.

Two polymorphisms in the promoter region of the TGFβ1 gene (G-800A and C-509T) were analysed. These polymorphisms may alter the rate of transcription by affecting the binding efficiency of transcription factor complexes. Nucleotide -800 of the TGFβ1 gene is located in a consensus sequence for the cAMP response element binding protein (CREB) family of transcription factors. An A at nucleotide -800 of the TGFβ1 gene, the lower producer allele, would be expected to decrease the DNA binding affinity of CREB family proteins, which may lead to a decrease in TGFβ1 expression (Kim *et al*, 1992). However, no significant differences in either genotype or gene frequency could be detected between ET patients and normal controls for either polymorphism, suggesting that the two promoter polymorphisms in the TGFβ1 gene do not have a role in the pathogenesis of ET.

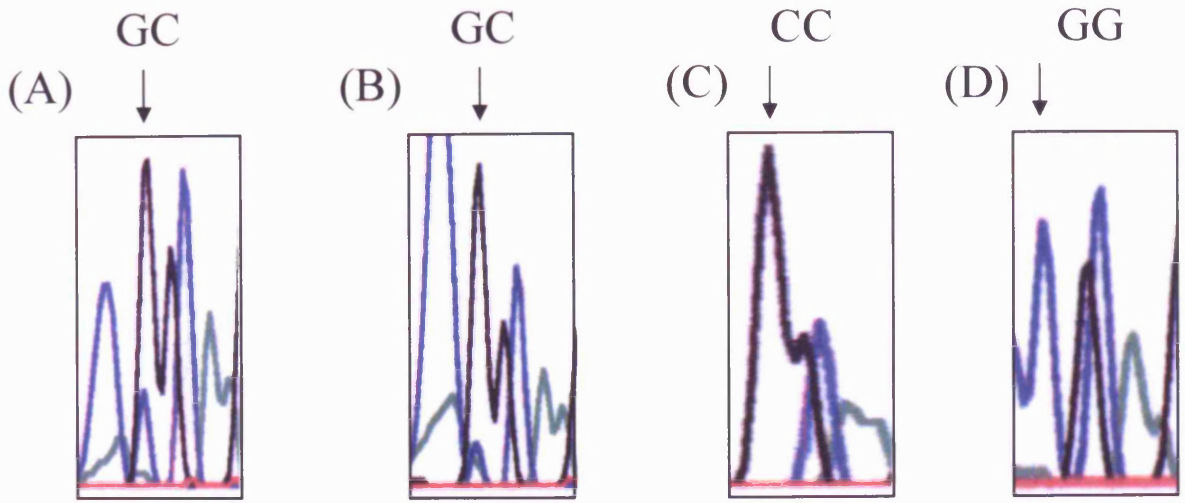

Figure 6.10
Sequences of two heterozygotes and each homozygote at nucleotide -174 of the IL-6 gene.
(A) is a heterozygote GC patient with a low intensity band at 108bp on ethidium bromide stained agarose gels, (B) is a heterozygote GC patient with a high intensity band at 108bp. (C) and (D) are homozygotes for CC and GG respectively.
Black trace represents C, blue trace represents G. Arrows indicate nucleotide -174

Two polymorphisms in the coding region of the TGFβ1 gene (T869C and G915C) were studied. Coding region polymorphisms may alter protein expression by mechanisms acting at the RNA or protein level. A sequence change may cause the mRNA to adopt a stem loop configuration, which would be expected to reduce the efficiency of translation. A less stable mRNA secondary structure may be produced which is more readily degraded, decreasing the availability of message which would lead to a decrease in protein expression. At the protein level, amino acid changes can lead to inefficient post-translational modification or disruption of signal sequence recognition which may reduce protein secretion rates from the cell, as can alterations in the signal cleavage site. However, the allele frequency and the percentage of each genotype in the ET patients and normal controls were not significantly different, suggesting that the two polymorphisms in the signal peptide encoding region of TGFβ1 are not involved in the pathogenesis of ET.

Undescribed polymorphisms or mutations may exist in the TGFβ1 coding or non-coding regions which may affect TGFβ1 release into the circulation. However, as the major store of TGFβ1 is the α-granules of MK and platelets, it may be that normal release of TGFβ1 from the increased MK and platelet load in ET patients accounts for the increase in serum/plasma TGFβ1.

In the promoter region of the IL-6 gene a G-174C polymorphism has been identified which affects plasma IL-6 levels. Alleles containing G are higher producer alleles and lead to increased circulating IL-6 compared to C containing alleles (Fishman *et al*, 1998). Binding of the glucocorticoid receptor, which is a transcriptional repressor, occurs around nucleotide -201 of the IL-6 gene (Ray *et al*, 1990). The G-174C polymorphism is close enough to nucleotide -201 to potentially influence binding efficiency of the glucocorticoid receptor. Also, a C at position -174 of the IL-6 gene creates a binding site for NF-1 in the gene promoter (Fishman *et al*, 1998). NF-1 has been shown to be a general repressor of transcription in HeLa cells and could therefore account for the decreased expression reported for C containing alleles (Rein *et al*, 1995).

No difference in allele frequency could be detected between ET patients and haematologically normal controls suggesting that the IL-6 polymorphism is not involved in ET. While the genotypes of the two groups were slightly different, this is probably due to small sample size rather than disease specificity. If the polymorphism was involved in ET it would be more likely to be involved in polyclonal pathogenesis. However, there was also

no correlation between genotype and clonality status in the ET patients, further suggesting that the G-174C polymorphism in the IL-6 gene in not involved in the pathogenesis of ET.

In conclusion, analysis of the G-800A, C-509T, T869C and G915C polymorphisms in the TGFβ1 gene and the G-174C polymorphism in the IL-6 gene has been unable to demonstrate any relationship between these polymorphisms and the pathophysiology of ET.

Chapter 7

Analysis of differential mRNA expression between platelets from an ET patient with monoclonal myelopoiesis and a haematologically normal control using representational difference analysis

7.1 Introduction

7.1.1 Investigating differential mRNA expression

The pathogenesis of ET may be extremely complex and could involve a number of genes, both known and/or previously undescribed. Mutations which affect gene function are likely to be important in disease pathology, but changes in gene expression levels, either gross or more subtle alterations, may also have a major role in disease pathology. A global approach was therefore adopted to study differences in gene expression between platelet mRNA obtained from ET patients and haematologically normal controls. A number of approaches are available to investigate differential gene expression, but 2 of the most frequently used are representational difference analysis (RDA) and microarrays. Microarrays utilize a vast number of defined oligonucleotide probes bound to a solid support medium which is then probed with fluorescently labelled cDNA. This technology is very powerful and able to identify large numbers of differentially expressed genes. However, results can be complex and array technology is unable to identify novel sequences. RDA is able to detect changes in mRNA expression between two populations without prior knowledge of sequence or specific genes. It is also extremely sensitive and has been able to identify known transcripts spiked into an RDA reaction which made up as little as 0.0005% of total mRNA (O'Neill & Sinclair, 1997).

In its original form RDA was used to identify differences between genomic DNA populations by a series of hybridization reactions and selective PCR amplification (Lisitsyn *et al*, 1993; Lisitsyn, 1995). This process was extensively modified to enable the analysis of differences in expressed mRNA populations (Hubank & Schatz, 1994) and further optimised to streamline the procedure and reduce the incidence of false positive results (Pastorian *et al*, 2000).

Two populations of RNA are prepared for an RDA procedure, the RNA population of interest is used as tester and the control RNA population is used as driver. Successive rounds of hybridization with an excess of driver, followed by selective PCR, amplify and enrich for differentially expressed genes, and dilution of the PCR products between rounds of hybridization subtracts non-differentially expressed genes (Figure 7.1). At the end of the process subtracted RDA products can easily be isolated by cloning, and identified by sequence analysis.

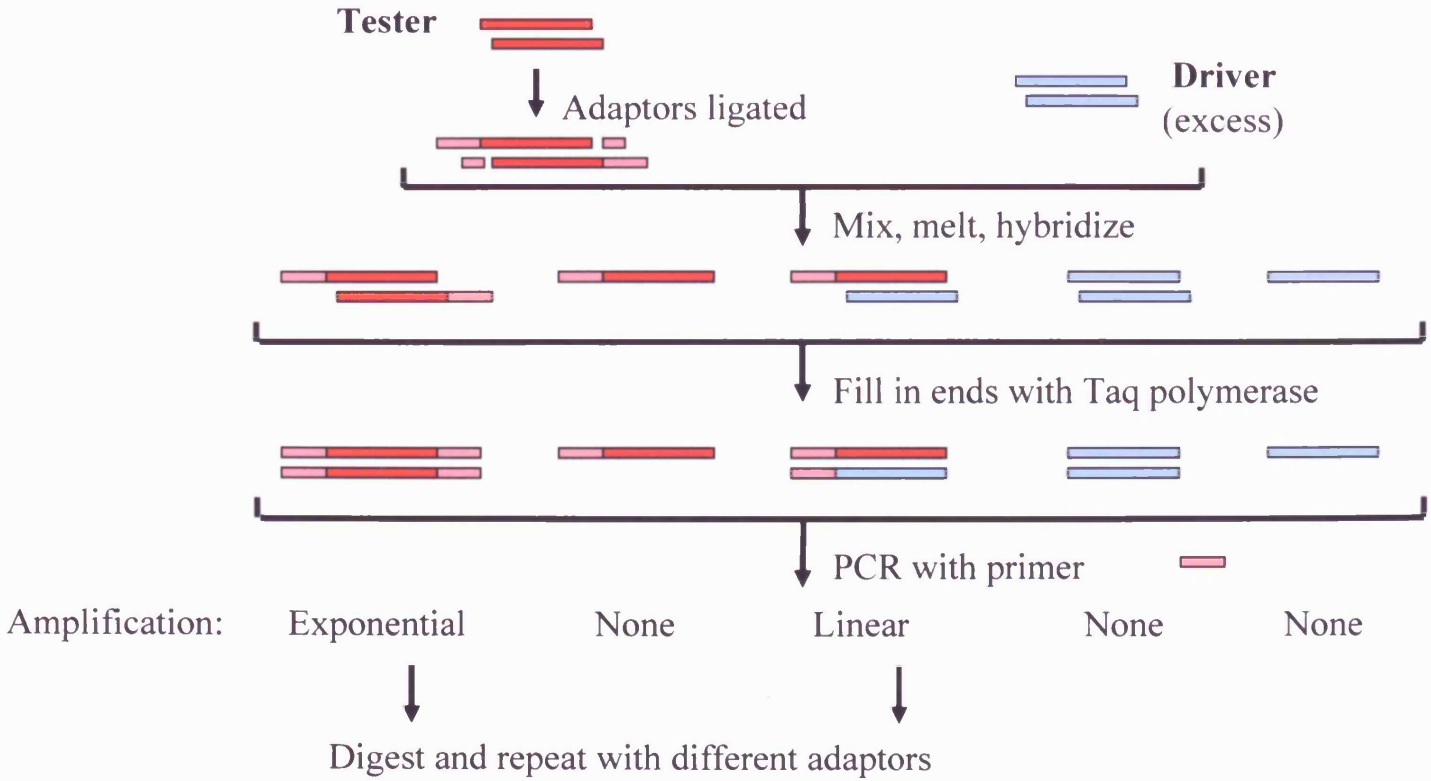

Figure 7.1
The RDA hybridisation and amplification procedure
Amplification can only occur when sequences are only, or more highly, expressed in tester. This leads to enrichment for these sequences in the difference products (Modified from Hubank and Schatz 1994).

RDA has been used extensively in cell lines to assess gene expression. For example, RDA was used to compare RNA from U937 cells transduced with the AML/ETO fusion protein, generated from the t(8;21) translocation in AML, to vector only transduced control cells. 26 novel genes whose expression levels were significantly modulated by AML/ETO induction were identified (Fliegauf *et al*, 2004). The effects of cytokine stimulation have also been investigated by RDA in cell lines. The myeloblastic cell line 32Dc13 was used to study the effect on RNA expression of IL-3 stimulation. Two aliquots of cells were prepared, one aliquot was starved for 24 hours without growth factors, the other aliquot was actively proliferating due to stimulation with 10% WEHI (conditioned media containing IL-3). A novel gene termed the small unstable apoptotic protein was shown to be up-regulated during apoptotic death due to IL-3 deprivation (Baker, 2003).

The differences between the starting populations of mRNA from cell lines should be solely due to how they have been manipulated for the experimental procedure. However, in primary cells the situation is more complex due to biological variation, which could cause non-disease related, inter-individual differences in gene expression. While the technique is optimised to minimise artefact, it is impossible to eradicate all technical problems, therefore the number of false positive clones would be expected to increase. Genes expressed at a very high level may not be sufficiently subtracted during the RDA procedure. Because of these problems, a number of screening steps are required to confirm that genes identified by RDA are truly differentially expressed and are disease specific. These screening methods are described in section 7.3.

However, RDA has been used successfully to identify disease-specific changes in mRNA levels in patient material. A commercially available variation of RDA (PCR Select^{TM} cDNA subtraction kit, CLONTECH), which uses a single round of hybridisation and amplification rather than the multiple rounds used by Hubank and Schatz (1994), was successfully used to isolate the PRV-1 gene from pools of granulocyte mRNA obtained from 5 PV patients and compared to normal controls (Temerinac *et al*, 2000). After extensive screening of the RDA products, the PRV-1 gene was identified and shown to be overexpressed in the granulocytes of PV patients compared to normal controls, secondary erythrocytoses and some ET patients.

7.1.2 Aim

The aim of the work described in this chapter was to compare global gene expression in platelet mRNA obtained from an ET patient and a normal control subject in order to identify genes which may be involved in the pathogenesis of ET, or which may be useful as a diagnostic marker for ET. After initial screening of the RDA products, the expression levels of one gene that was shown to be differentially expressed were quantified in platelet mRNA from a cohort of ET patients compared to a number of normal controls, patients with other cMPDs and patients with reactive thrombocytosis.

7.2 Overview of the generation of difference products (DP)

The RDA procedure used to produce clones for screening was carried out by Dr. R. Gale and is summarised in Figure 7.2 (A and B). Platelet RNA was obtained from one ET patient with monoclonal myelopoiesis (XCIP result by HUMARA; T-cells 50%:50% expression of the 2 alleles, neutrophils 100%:0%) and one normal control. The SMART^{TM} PCR cDNA synthesis kit (CLONTECH) was used to initially amplify polyA$^+$ mRNA to enrich for full length cDNAs (Figure 7.3). Representations were prepared from cDNA from each sample (M_{rep} = representation prepared from platelet RNA obtained from one ET patient with monoclonal myelopoiesis, N_{rep} = representation prepared from platelet RNA from one normal control) by *Dpn*II digestion of the double stranded cDNA followed by PCR amplification. The representations were then used either as driver or tester in the initial hybridization reaction at a driver to tester ratio of 4:1. Two difference products (DP1) were generated by selective PCR: the forward reaction nM1 (N_{rep} driver, M_{rep} tester – enriched for sequences only, or more highly expressed, in the monoclonal ET patient sample) and the reverse reaction mN1 (M_{rep} driver, N_{rep} tester – enriched for sequences only, or more highly expressed, in normal control sample). During the PCR step only differentially expressed sequences should have been exponentially amplified (Figure 7.1). A second round of hybridization was carried out to further enrich for differentially expressed genes. DP2 products were generated by hybridizing driver to DP1 products at a ratio of 16:1 (Figure 7.4). After the final PCR of the RDA process, the DP2 products were cloned into the TOPO-2 vector (Invitrogen) and 48 clones isolated for both the forward

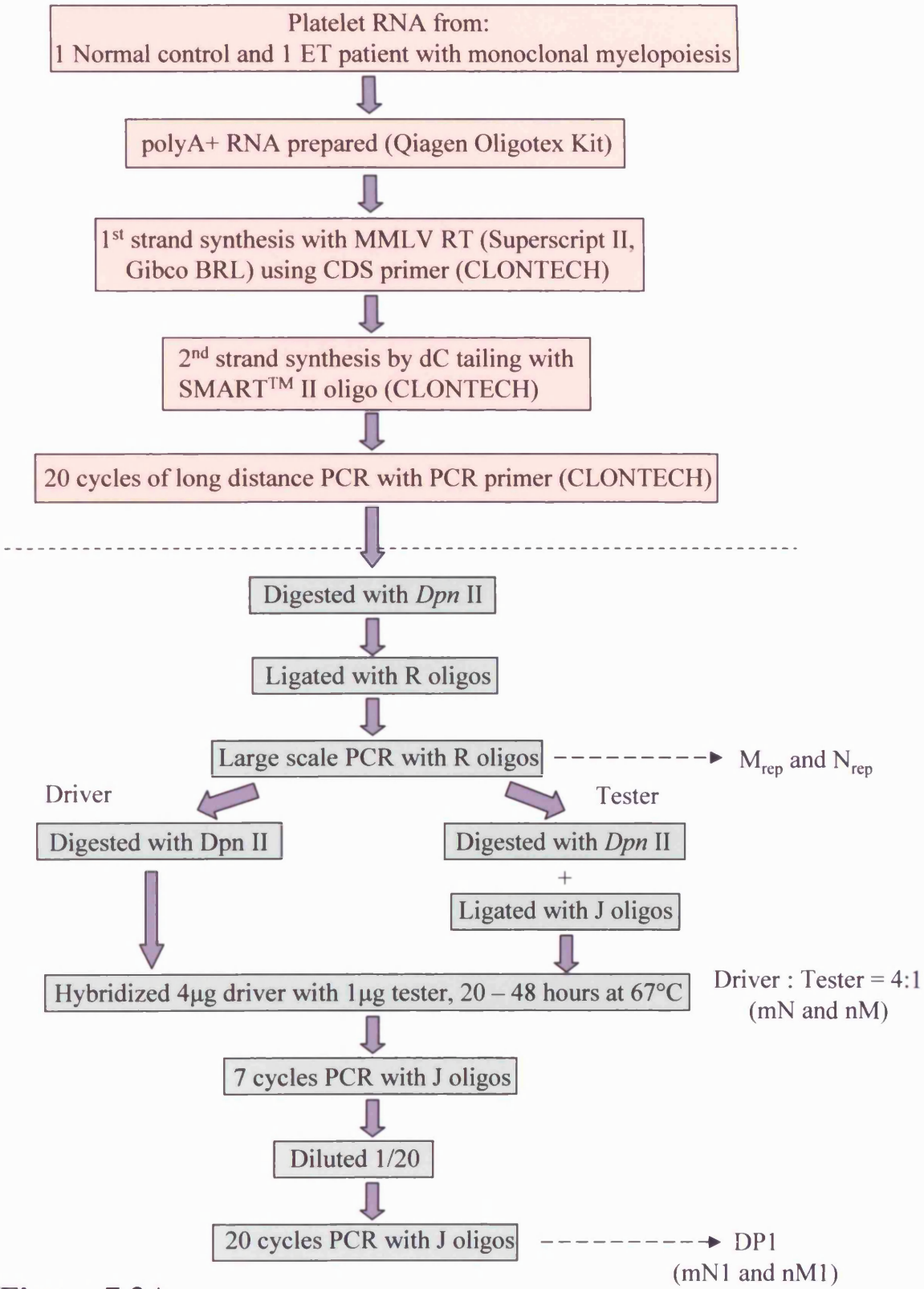

Figure 7.2A

Generation of double stranded cDNA, representations and difference product 1 (DP1). M_{rep} = monoclonal ET representation, N_{rep} = normal control representation, m = monoclonal ET driver, M = monoclonal ET tester, n = normal control driver, N = normal control tester.

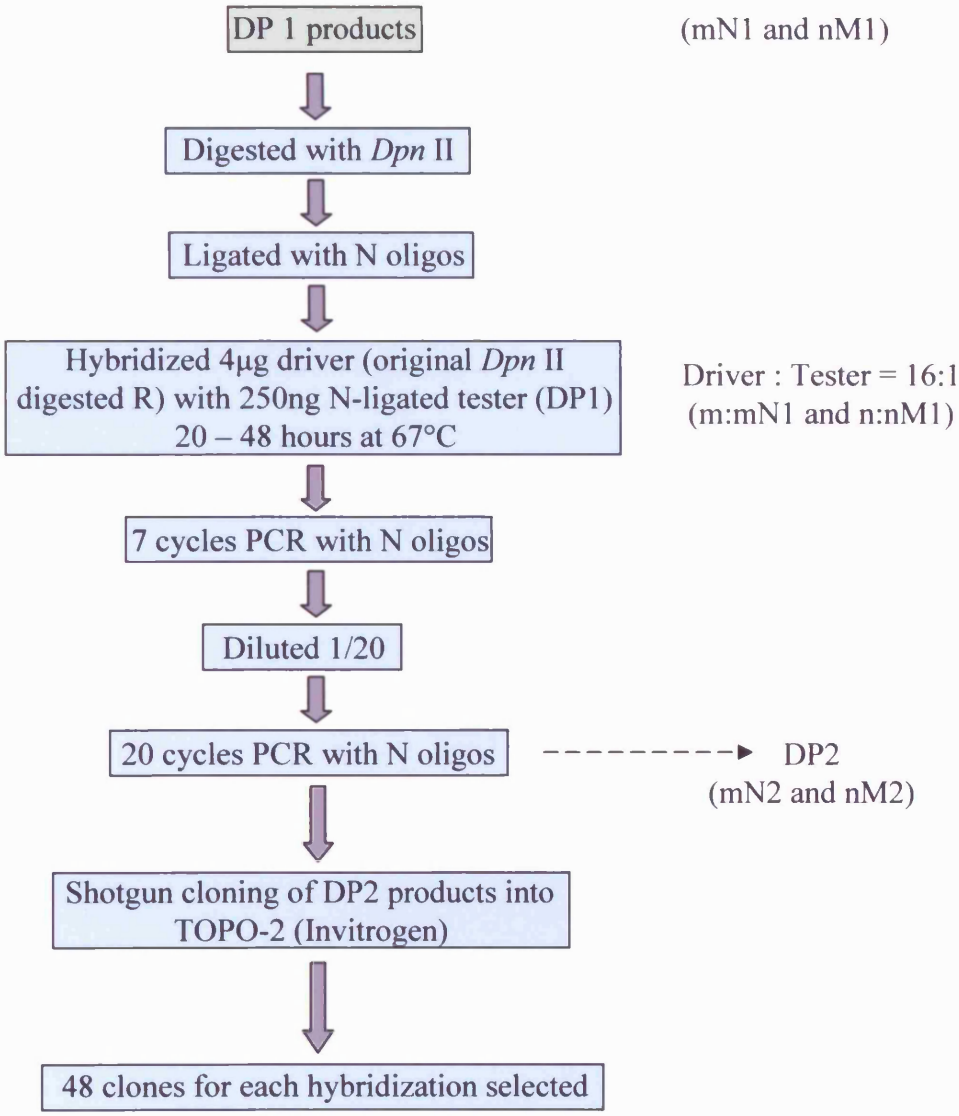

Figure 7.2B
Generation of difference product 2 (DP2) and cloning of DP2 products. m = monoclonal ET driver, M = monoclonal ET tester, n = normal control driver, N = normal control tester. Protocol based on CLONTECH SMART™ PCR, Hubank and Schatz (1994) and Pastorian *et al* (2000)

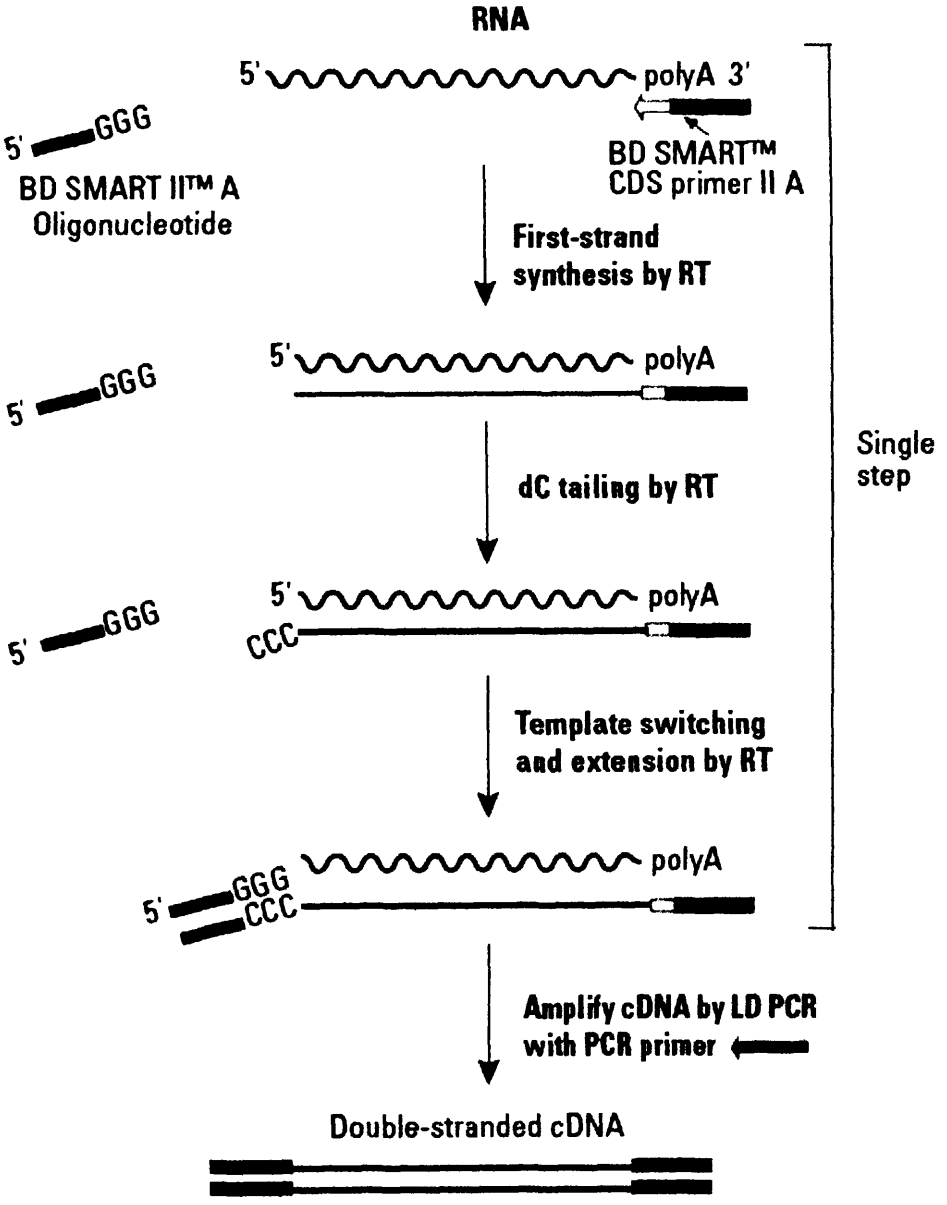

Figure 7.3
SMART™ PCR cDNA synthesis
(From SMART™ PCR cDNA synthesis kit user manual 2001, CLONTECH)

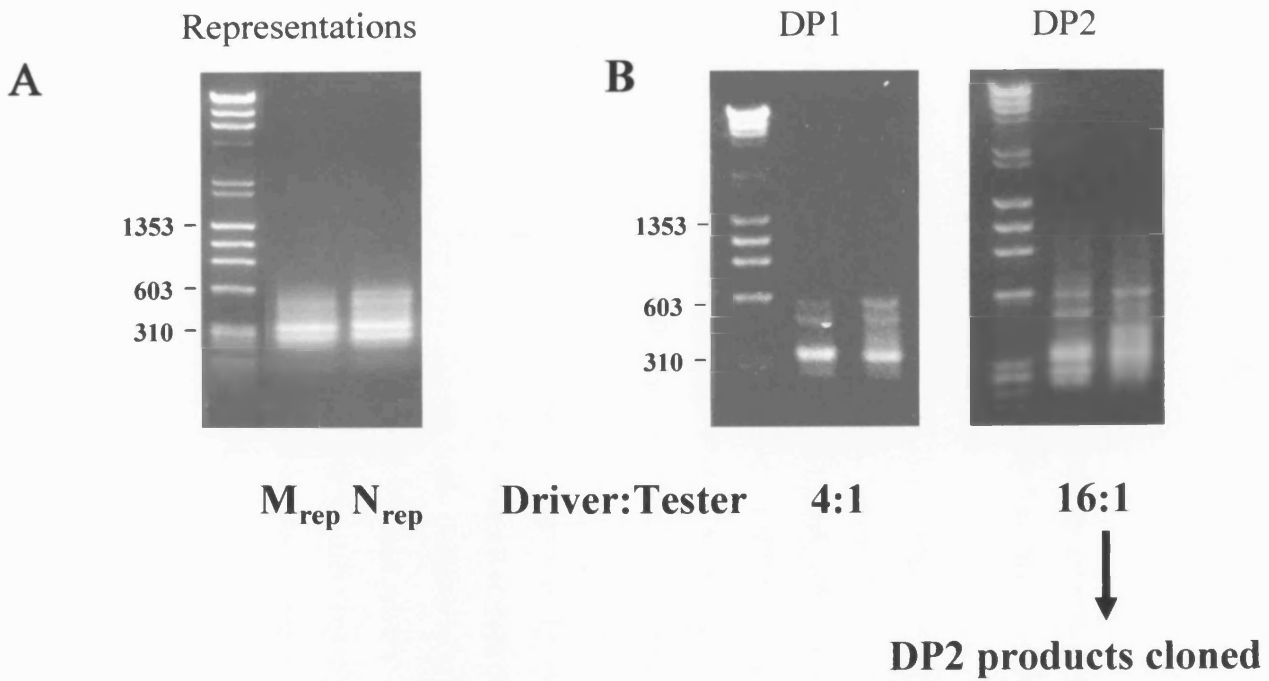

Figure 7.4
Gel images of driver, tester and difference products
M_{rep} is unsubtracted representation produced from an ET patient with monoclonal myelopoiesis, N_{rep} is unsubtracted representation produced from normal control. Driver : Tester ratios are given for DP1 and DP2.
(Produced by Dr R. Gale)

(nM2) and reverse (mN2) RDA reaction. All work in this chapter was carried out from these 96 clones.

7.3 Screening of DP2 Products

7.3.1 Dot blots

Principle Each clone produced by the RDA procedure was screened by hybridizing to both the unsubtracted representations and to the subtracted difference products produced by the forward and reverse reactions. The likelihood that any clone represents a differentially expressed gene can then be estimated from the binding patterns produced. For nM2 products, the forward subtraction is nM2, the reverse subtraction is mN2, and tester and driver are M_{rep} and N_{rep} respectively. All clones which represent differentially expressed genes should hybridize with the forward subtraction probe (i.e. nM2 clones must be positive with the nM2 probe). Most clones which are also negative with the reverse subtraction, positive with tester and negative with driver are likely to be fragments of differentially expressed mRNAs (Table 7.1 A). Clones which are negative with the reverse subtraction and both tester and driver are likely to be good candidates for differential expression (Table 7.1 B). Clones which are negative with the reverse reaction and positive with both tester and driver may also be from differentially expressed genes, but they may be artefact due to high expression in both ET and normal samples (Table 7.1 C). Clones which are positive with all probes may be from differentially expressed genes if the degree of positivity is higher with the forward subtraction and tester probed membranes (Table 7.1 D). Any other hybridization patterns are not suggestive of differential expression.

Method PCR products were obtained from the 96 cloned DP2 products using 35 amplification cycles with the N oligo and Bioline reagents (Chapter 2, 2.2.7) and were analysed for size by agarose gel electrophoresis. These products were then individually hybridized to 4 probes, the monoclonal representation (M_{rep}), the normal control representation (N_{rep}), nM2 product and mN2 product. One µl fresh PCR product was mixed with 1µl 0.6M NaOH to denature the DNA and aid binding to the nylon membrane, before being spotted onto Hybond N^+ nylon membrane (Amersham Pharmacia). Four membranes were prepared, each containing 96 dots in a 96 well plate format, 48 from nM2 clones and

	Probe used				
	Forward Reaction	Reverse Reaction	Tester	Driver	Likelihood of being differentially expressed
A	+	-	+	-	>95% of these clones will be differentially expressed
B	+	-	-	-	High probability of differential expression
C	+	-	+	+	Difficult to interpret, could be differentially expressed
D	++	+	+	+/-	Can be differentially expressed, according to fold difference of positive clones
	+	+	+	+	Rarely differentially expressed
	-	-	-	-	Rarely differentially expressed

Table 7.1
Interpretation of dot blot results.
(Modified from CLONTECH PCR-Select^{TM} differential screening kit user manual, 2001).

48 from mN2 clones. The membranes were neutralised for 4 minutes with 1.5M NaCl, 0.5M TrisHCl pH 7.2 and 0.001M EDTA (neutralising solution), before being fixed for 20 minutes in 0.4M NaOH. After fixing, the membranes were rinsed in 5ml 5x SSPE for 45 seconds (20 x SSPE contains 3M NaCl, 0.2M $NaH_2PO_4.H_2O$, 20mM EDTA).

To prepare for probe hybridization, the membranes were incubated for 2 hours at 65°C with 10ml hybridization solution (10x Denhardt's solution: 0.25% Ficoll, 0.25% polyvinylpyrrolidone, 0.25% BSA, and 4 x SSPE, 0.1% SDS, 10mg/ml denatured, sheared single stranded DNA [ssDNA]) to reduce non-specific DNA binding. During this pre-hybridization, the four probes were prepared.

The random primed DNA labelling kit (Roche) and [α-^{32}P]dCTP (0.37MBq/µl, Amersham Pharmacia) were used to label the probes. Fifty ng of M_{rep}, N_{rep}, nM2 or mN2 was incubated with de-ionised water to 10µl final volume at 95°C for 5 minutes and transferred directly to ice. Once cooled, 2µl reaction mixture (containing random hexanucleotide primers in 10 x reaction buffer), 1µl Klenow fragment, 1µl each of dATP, dGTP, dTTP and 4µl [α-^{32}P]dCTP were added and the reaction incubated at 37°C for 1 hour. During this incubation a complementary DNA strand is synthesised by the Klenow enzyme from the 3' terminus of the hexanucleotide primers, incorporating isotope labelled cytosine. The probes were then centrifuged through Sephadex G50 columns for 6 minutes at 2000rpm to remove unincorporated [α-^{32}P]dCTP. To ensure an equal amount of labelled probe was used for each hybridization, probes were counted by mixing 1µl probe with 4.5ml scintillation fluid and the disintegrations per minute (DPM) measured using a scintillation counter. One hundred µl ssDNA (2mg) was added to an aliquot containing $25x10^5$ DPM each probe and incubated at 95°C for 5 minutes, quenched on ice and then added to 5ml hybridization solution. Pre-hybridized membranes were then incubated in this solution for 17 hours at 65°C.

After incubation the membranes were washed in 500ml 2xSSPE, 0.1% SDS for 15 minutes at room temperature to remove non-specifically bound probe. This wash was repeated and then followed by a further wash for 30 minutes at 65°C. Each membrane was then monitored for presence of isotope using a Geiger counter. If isotope levels were in excess of approximately 200cpm then a higher stringency wash was carried out using 0.2xSSPE, 0.1% SDS for 30 minutes at 65°C. The membranes were then covered in Saran wrap and exposed to autoradiography film (Hyperfilm, Amersham Pharmacia) for 1 hour at -80°C.

Results The sizes of the clones produced by the RDA reaction ranged from approximately 200bp to 800bp. To establish which clones were likely to be expressed differentially all spots were scored according to dot intensity as either highly positive (++), positive (+), difficult to determine positivity (+/-) or negative (-) (Table 7.2). For example, of the nM2 products probed with N_{rep}, D9 was scored +/-, D10 was scored ++, D11 was scored + and D12 was scored – (Figure 7.5). Of the 96 clones from both the forward and reverse reactions, 15 were selected which had a high probability of being differentially expressed (Table 7.1 A, B), 4 from nM2: A8, B4, C1, and D2 (Figure 7.5) and 11 from mN2: A3, A4, A8, B8, B9, B10, B11, C11, D8, D10 and D11 (Figure 7.6). Eight clones were identified which could be differentially expressed but this was less likely than those above, all 8 were clones from mN2 (A9, A11, B1, C7, C10, C12, D9 and D12). Therefore, a total of 23 clones were further screened for differential expression using Virtual Northern blot analysis, 4 from nM2 and 19 from mN2.

7.3.2 Virtual northern blots

Principle Virtual northern blots are similar to northern blots, but use amplified cDNA rather than RNA. In this instance, the M_{rep} and N_{rep} were individually probed with the cloned DP2 products identified by dot blot as being from differentially expressed genes. This allowed a direct comparison of the levels of each clone in the initial representations, decreasing the subjectivity of scoring each clone due to non-specific hybridization in the dot blot analysis.

Method The 23 clones selected from dot blot assay were analysed by virtual northern blots. Agarose gels (1.5%) were prepared and loaded with 200ng each of M_{rep} and N_{rep}, and the samples separated by gel electrophoresis. The gels were placed in 100ml denaturation buffer (1.5M NaCl, 0.5M NaOH) for 15 minutes at room temperature to ensure the DNA was fully denatured, and then rinsed in de-ionised water for 2 minutes. A capillary transfer system was used to transfer the DNA to Hybond N+ nylon membrane (Amersham Pharmacia). The membrane was placed on the agarose gel and transfer buffer (1.5M NaCl, 0.25M NaOH) allowed to pass through them both, by capillary action, over the course of 17 hours. The membranes were then washed in 100ml 2xSSPE at room temperature for 2 minutes to remove any adherent agarose and stored in 10ml hybridization solution.

Each of the 23 candidate clones were separately used to probe the membranes. Probes were prepared from the original PCR products generated from cloned DP2 products.

(A)

	A	B	C	D	
A1	-	-	-	++	
A2	-	+	-	+	
A3	-	-	-	+/-	
A4	+	-	-	+/-	
A5	+	-	+/-	+/-	
A6	-	-	-	-	
A7	-	+	-	-	
A8	+/-	-	-	-	*
A9	-	-	-	+	
A10	+/-	++	-	++	
A11	-	-	-	+/-	
A12	-	+/-	+/-	+/-	
B1	+	+	-	++	
B2	-	-	-	-	
B3	-	-	-	-	
B4	+/-	-	-	-	*
B5	-	-	-	+/-	
B6	+/-	+	+	-	
B7	-	+	-	-	
B8	-	+/-	-	-	
B9	-	-	-	+/-	
B10	+	+	+	++	
B11	-	-	-	-	
B12	-	-	-	-	

	A	B	C	D	
C1	+	-	-	-	*
C2	+	+	-	+/-	
C3	+	+/-	-	++	
C4	+	+/-	-	+	
C5	-	-	-	+	
C6	-	+	-	+/-	
C7	-	-	-	+/-	
C8	+	+	-	+	
C9	-	-	-	+/-	
C10	-	+	-	+	
C11	-	+	-	++	
C12	-	-	-	-	
D1	+/-	-	-	+	
D2	+/-	-	-	-	*
D3	-	-	-	+/-	
D4	-	-	-	+/-	
D5	-	+/-	+/-	+/-	
D6	-	-	-	+/-	
D7	-	-	-	+/-	
D8	+/-	+	-	+	
D9	-	-	-	+/-	
D10	+/-	++	+/-	++	
D11	-	+	-	+	
D12	-	-	-	-	

(B)

	A	B	C	D	
A1	-	-	+	+	
A2	+	-	+	+	
A3	+	-	+	-	*
A4	++	-	+/-	-	*
A5	+	+	++	-	
A6	-	+	+	-	
A7	-	-	+	+/-	
A8	+/-	-	+/-	-	*
A9	+/-	+/-	++	+/-	†
A10	-	-	-	-	
A11	+/-	+/-	++	+/-	†
A12	-	-	++	+	
B1	+	+/-	+	+	†
B2	-	-	-	-	
B3	+	+	+/-	-	
B4	-	-	-	-	
B5	-	-	+/-	-	
B6	-	-	-	-	
B7	-	+	-	+	
B8	+/-	-	+/-	-	*
B9	+/-	-	+/-	-	*
B10	+	-	++	-	*
B11	+	-	+	-	*
B12	++	-	+	++	

	A	B	C	D	
C1	-	-	+	-	
C2	-	-	+/-	-	
C3	-	++	++	-	
C4	++	++	++	+	
C5	++	+	+/-	-	
C6	+	++	+/-	+	
C7	+	+/-	+	+	†
C8	+/-	+/-	+	-	
C9	+/-	+/-	+	-	
C10	+	+/-	++	-	†
C11	+	-	++	-	*
C12	++	-	++	+	†
D1	-	+	+	-	
D2	+/-	+	++	-	
D3	-	+/-	++	-	
D4	+/-	++	++	+/-	
D5	++	++	+	+/-	
D6	+	-	-	+	
D7	+	+/-	+	+	
D8	++	-	+	-	*
D9	++	+/-	+	-	†
D10	+/-	-	++	-	*
D11	++	+/-	++	-	*
D12	++	-	++	+	†

Table 7.2

Dot blot scoring for (A) nM2 clones and (B) mN2 clones

Each set of 48 clones was probed with:

A - Forward subtraction, B - Reverse subtraction, C - Tester and D - Driver

* = High probability of differential expression † = Could be differentially expressed

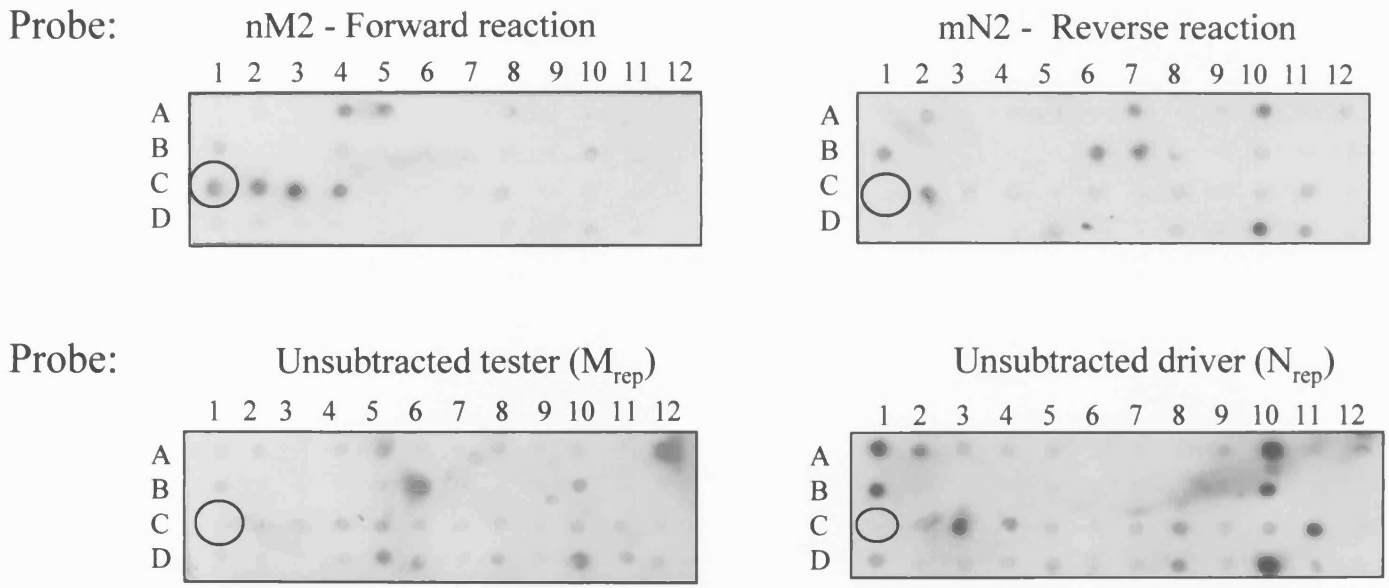

Figure 7.5
Dot blots of 48 nM2 products
Clones created using the M_{rep} as tester and the N_{rep} as driver for generation of DP1, and the N_{rep} as driver for DP2. A good candidate clone is circled: positive for the forward subtraction, and negative for all other probes

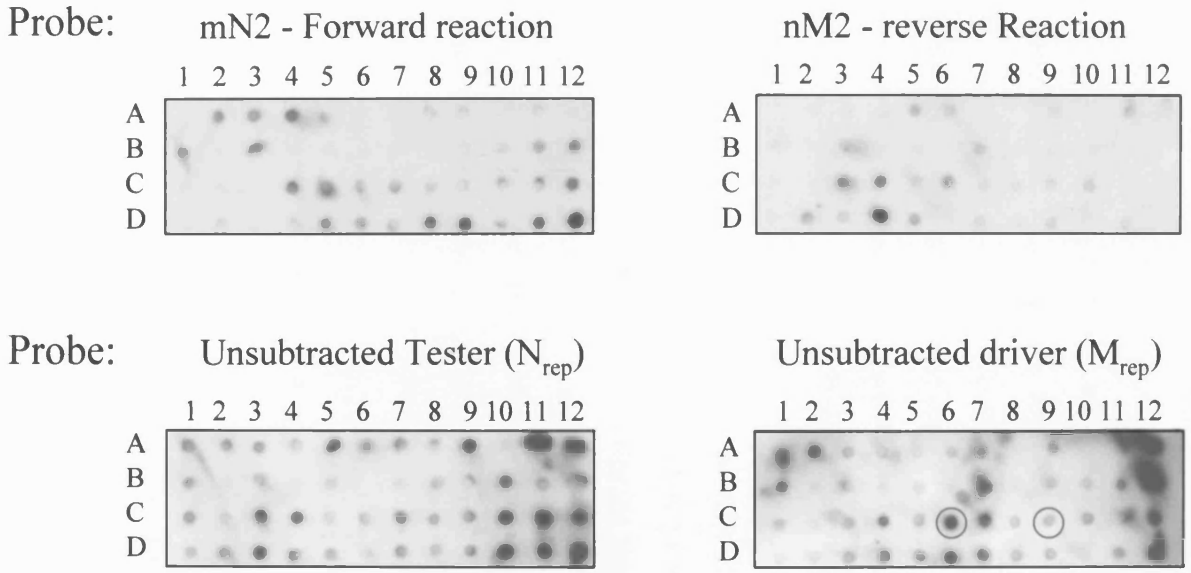

Figure 7.6

Dot blots of 48 mN2 products

Clones created using the N_{rep} as tester and the M_{rep} as driver for generation of DP1, and the M_{rep} as driver for DP2. For the blot probed with unsubtracted driver (M_{rep}), C6 (green circle) shows a positive result, C9 (red circle) shows a negative result with high background signal

Each PCR product was diluted 1/500 and re-amplified using 30 cycles of PCR with the N oligo at an annealing temperature of 64°C as described in chapter 2 (2.2.7). PCR products were isotope labelled with α-^{32}P-dCTP by random primed DNA labelling as described in section 7.3.1. Probes were cleaned through Sephadex G50 columns and hybridized to the membranes as outlined in section 7.3.1. A high stringency wash was required to remove non-specifically bound radiolabelled probe, therefore the final wash was carried out using 500ml 0.1xSSPE, 0.1% SDS for 30 minutes at 65°C.

Results For 10 of the 23 clones used as probes in the virtual northerns, the relative intensity of the bands in M_{rep} and N_{rep} appeared to be equal. Therefore these clones were likely to be false positives and were not further studied. Thirteen of the 23 clones investigated by virtual northern analysis appeared to have bands of variable intensity in the M_{rep} and N_{rep} samples and were therefore likely to be differentially expressed. The band in M_{rep} was of higher intensity than the band in N_{rep} when all 4 clones selected from nM2 were used as probes (A8, B4, C1 and D2). This suggested that these clones represented genes which had a higher expression level in platelet RNA from the monoclonal ET patient than the haematologically normal control. When blots were probed with clones selected from mN2, the band in N_{rep} was of higher intensity than the band in M_{rep} for 9 clones (A3, A4, A8, B8, B9, B11, C11, D8, D12), suggesting that these clones represented genes which had a lower expression level in platelet RNA from the monoclonal ET patient than the haematologically normal control. Three representative samples are shown in Figure 7.7, clone mN2 A3 had a band of lower intensity in the M_{rep} compared to N_{rep}, clone nM2 C1 had a band of higher intensity in the M_{rep} compared to N_{rep}, and clone mN2 D10 had bands of similar intensity in both M_{rep} and N_{rep}.

7.3.3 Sequencing of differentially expressed genes

Method The 13 clones which appeared to be differentially expressed by virtual northern analysis were sequenced. The DP2 PCR products had the same primer annealing sites at both ends of the DNA fragment as a result of the RDA process (the N oligo). However, sequencing reactions require unidirectional linear amplification, therefore each product was cloned into the TOPO T-Cloning vector as described in chapter 2 (2.2.10) using 0.5µl fresh PCR product (prepared as in 7.2.3). The TOPO vector contains M13 primer sites close to the insertion site, one annealing site for the M13F(-20) primer and one annealing site for the –M13R primer Table 7.3. PCR was carried out using these two

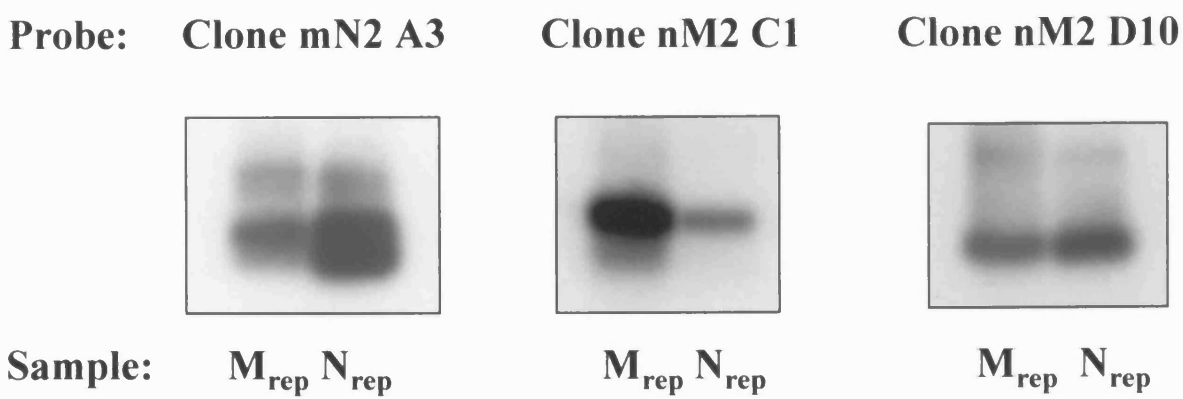

Figure 7.7
Virtual northern analysis of mN2 and nM2 clones
Three representative clones selected from dot blot screening are shown. Bands produced with mN2 clone A3 and nM2 clone C1 were of variable intensity between M_{rep} and N_{rep}. Bands produced with nM2 clone D10 were not thought to be of variable intensity.

	Forward primer	Reverse primer	Fragment size (bp)	Annealing Temperature (°C)
RANTES	CTGTACTCCTCATTGCTACTG	CTACCACACAGCAGCAGTTAC	390	62
FLP	TCCAGGACATCAAGAAGCCAG	TCAGAAGGCTCTTAGTCGTGC	303	60
CD32	CATCATGCTGAGGTGCCACAG	TTGGGCAGCCTTCACAGGATC	360	62
N-arginine dibasic convertase I	TGCTGTGGTCAAAGCTGACTG	GAAGTACTTCCTCTGGACATG	403	60
Cytochrome C Oxidase II	TCTGCTTCCTAGTCCTGTATG	TTAGACGTCCGGGAATTGCAT	437	60
12s rRNA	ATCCCCGTTCCAGTGAGTTCA	TGTTGAGGTTTAGGGCTAAGC	409	60
Cytochrome b	AGGAGGCTACTCAGTAGACAG	GATCGGAGAATTGTGTAGGCG	353	60
GAPDH	GCCGAGCCACATCGCTCAGA	GAGGCATTGCTGATGATCTTG	471	62
M13 primers	CAGGAAACAGCTATGAC	CTGGCCGTCGTTTTAC	V	50
R131H primers	TCCAGAATGGAAAATCCCAGAAATTCTAGA	GACCTCCATGTAGGCCCATGTG	231	66

Table 7.3
The primer sequences used to screening RDA products

The oligonucleotides used for RDA, screening and polymorphism analysis. All primers shown in 5'→3' direction, mismatch primer shows altered base underlined. For the M13 PCR reaction -M13R is the reverse primer, M13F(-20) is the forward primer.
V = variable size PCR product.

primers for 35 amplification cycles using Bioline reagents. The PCR products were cleaned using QIAquick PCR purification kit (Qiagen), cycle sequencing carried out using the M13F(-20) primer and samples loaded to the ABI310 genetic analyser as described in chapter 2 (2.2.9). Approximately 100bp of the resultant sequence was entered into a nucleotide-nucleotide BLAST (blastn) search (http://www.ncbi.nlm.nih. gov/ BLAST/).

Results The 4 clones identified from nM2 were shown to be fragments from 4 different genes: Clone A8 was a fragment from the Regulated upon Activation Normal T cell Expressed and Secreted (RANTES) gene, clone C2 was a fragment from the CD32 (FcγRIIA) gene, clone C4 was a fragment of the mitochondrial gene 12S rRNA and clone D2 was a portion of the Ferritin Light Polypeptide gene (FLP) (Table 7.4). The 9 clones selected from mN2 were fragments of 4 different genes: B8 was a fragment from the N-arginine dibasic convertase I (NRDI) gene, clones A4, B9, C11, D8 and D12 were fragments from the mitochondrial 12S rRNA, previously identified from clone C4 of the nM2 forward reaction, clones A3 and B1 were fragments from Cytochrome C Oxidase subunit II and clone A8 was from the cytochrome b gene (Table 7.4).

The sequences generated by all 13 clones had >95% homology to previously described genes. Of the four clones selected from nM2 products, 3 were fragments from genomic genes (RANTES, CD32, FLP) and one was a fragment from a mitochondrial gene (12S rRNA). Only one clone selected from the mN2 products was a fragment from a genomic gene (NRD1), the other 9 clones were fragments from 3 mitochondrial genes (12S rRNA, Cytochrome C Oxidase subunit II and cytochrome b).

7.3.4 Variable cycle number PCR

Principle The 2 major disadvantages with the RDA technique when used in primary cells are the propensity to amplify sequences which are expressed at high levels in both starting populations, irrespective of differences, and an inability to select only for disease-specific differences. As with all techniques used to assay differential expression, inter-individual differences will generate clones in the same way as disease-specific differences. To investigate whether the difference in the expression of the seven genes identified by screening RDA products was disease-specific, RT-PCR was carried out on a small number of patient and control samples and the expression levels of each gene between the two groups compared. The PCR reaction was carried out using a variable number of amplification cycles for each gene. This was because it was important that the PCR

	Clone	Number of bases entered into search	Gene	Sequence identifier	Homology (%)
nM2	A8	106	RANTES	NM 002985.2	97
	C2	131	CD32	XM 033957.3	94
	C4	98	12S rRNA	AY 012136.1	97
	D2	99	FLP	XM_050465.1	98
mN2	B8	97	NRD1	XM_001517.5A4	98
	A4	98-136	12S rRNA	AY 012136.1	>95
	B9				
	C11				
	D8				
	D12				
	A3	124-136	Cytochrome C oxidase subunit II	NC 001807.3	99
	B11				
	A8	96	Cytochrome b	NC 001807.3	98

Table 7.4
Sequence information for the 13 clones selected from screening of the RDA DP2.

products were obtained from the linear phase of the reaction, and that bands on the agarose gels were not saturated, which may lead to overrepresentation of a gene expressed at a low level, potentially masking any differential expression.

Method RNA was obtained from purified platelet samples from 3 ET patients with clonal myelopoiesis and 3 haematologically normal control subjects as shown in chapter 2 (2.2.1, 2.2.3). RT reactions were performed using 1μg RNA of each sample (Chapter 2, 2.2.6). Two μl of the RT products were added to the PCR as cDNA template and a hot start was used with Bioline reagents as described in chapter 2 (2.2.7). The FLP, CD32, RANTES and Cytochrome b genes were investigated using 22 and 27 cycles of amplification, the Cytochrome C Oxidase subunit II gene was investigated using 25 and 30 amplification cycles, and the 12S rRNA gene was investigated using 15 and 20 PCR amplification cycles. The forward and reverse primers used to investigate the expression of each gene, the annealing temperature used and the expected PCR product sizes are detailed in Table 7.3. PCR products were electrophoresed through 1.5% agarose gels and visualised with ethidium bromide staining.

Results The intensity of the RT-PCR product for each gene in 3 ET patients with clonal myelopoiesis was visually compared to 3 normal control samples. RANTES PCR products appeared more intense in the ET patients than normal controls using 22 cycles (Figure 7.8). Differences in intensity were still evident when 27 amplification cycles were used but they were less pronounced due to saturation. PCR products for the FLP gene were very faint using 22 cycles of amplification and differential expression was difficult to determine. However, when 27 amplification cycles were used the bands produced from ET patients with clonal myelopoiesis appeared to be more intense than bands from normal controls. This was also the case for CD32 PCR products, where only samples amplified for 27 cycles of PCR were evaluable, and ET patients with clonal myelopoiesis had more intense bands than normal controls. None of the 3 mitochondrial genes appeared to be differentially expressed between the two groups (Figure 7.8), even where the numbers of amplification cycles used was low enough to ensure the absence of saturation which may have masked differences in expression.

The NRD1 RT-PCR product was of low intensity and had multiple bands. While the intensity of the product could be increased by carrying out more amplification cycles, this also increased the number of extra bands produced. These bands were still present after optimization for magnesium ion concentration and annealing temperature. As these extra

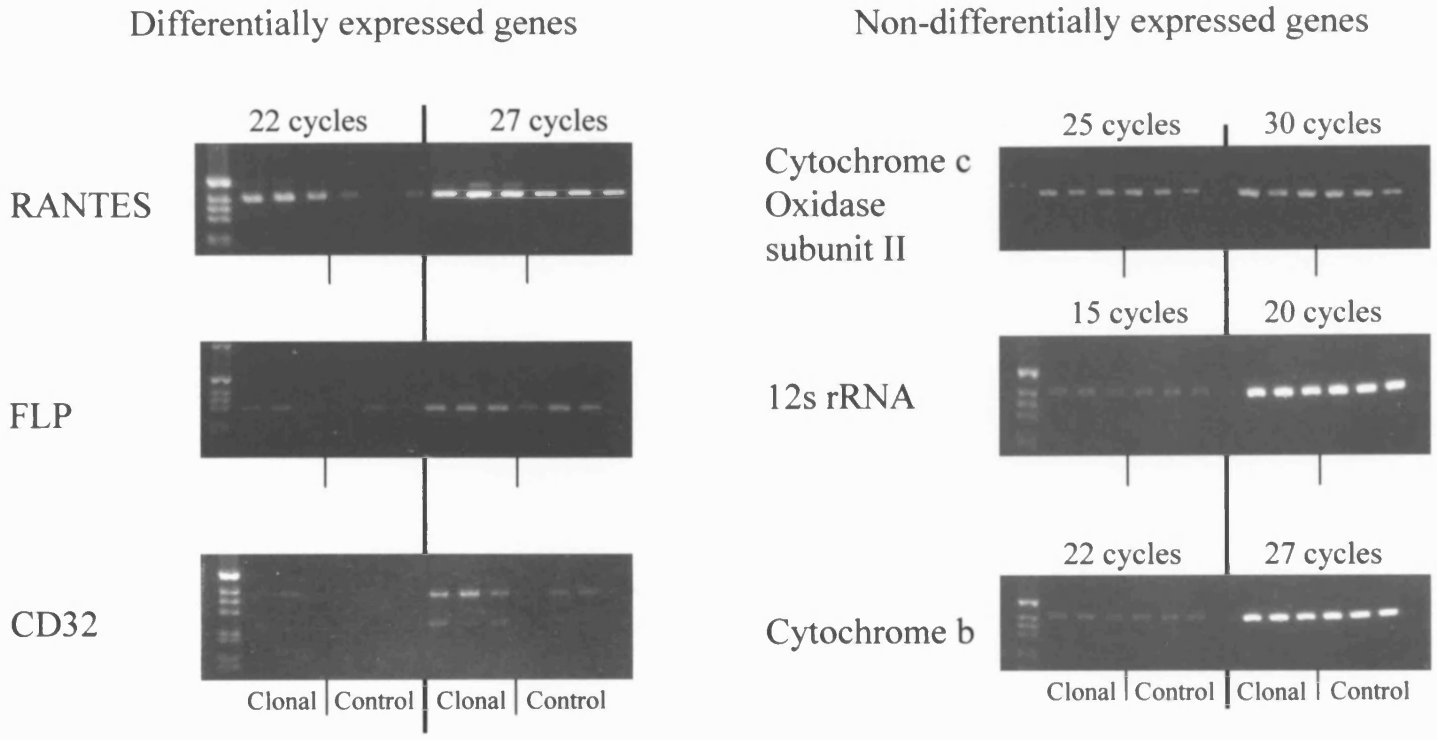

Figure 7.8
Variable cycle number RT-PCR
The six genes investigated by variable cycle number RT-PCR are shown for 3 ET patients with clonal myelopoiesis and 3 haematologically normal controls. Numbers of amplification cycles are shown for each gene

bands made it difficult to assess expression levels between different samples and may have interfered with subsequent quantification, NRD1 was not further investigated.

Therefore, while no difference in the expression of the 3 mitochondrial genes was apparent between the two groups, 3 somatic genes were identified which appeared to be overexpressed in platelet mRNA obtained from ET patients with clonal myelopoiesis compared to platelet mRNA from normal control subjects.

7.4 Analysis of CD32

7.4.1 Introduction

Of the three genes with an apparently increased expression in clonal ET patients, CD32 was thought to be the most likely to be involved in the pathogenesis of ET and so its expression level in platelet mRNA was further investigated. CD32 is an Fcγ receptor expressed on the surface of platelets and is known to be involved in thrombus formation. Immunoglobulin G (IgG) can interact with various membrane receptors via their Fc region. These Fc receptors (FcγR) occur in three different groups depending on structural and functional criteria. CD32 is a group II FcγR, FcγRIIa, the only FcγR present on platelets (Parren *et al*, 1992; Ravetch & Kinet, 1991). It has been shown to associate with the von Willebrand factor (vWF)/GpIb-IX-V complex and mediate platelet activation and thrombus formation (Sullam *et al*, 1998; Sun *et al*, 1999).

7.4.2 Method

To assess whether the increased levels of expression of CD32 seen in the preliminary RT-PCR experimental data (Section 7.3.4) were reflective of disease-specific changes, CD32 expression was investigated in platelet mRNA from 3 patient groups and normal controls using a radioactively labelled semi-quantitative multiplex RT-PCR (SQ-RT-PCR). Two primer sets were used in a single tube PCR reaction, one primer set to amplify a portion of the CD32 gene, the other to amplify a portion of a control gene. The relative intensity of the CD32 PCR product was compared to that of the control gene to obtain a relative ratio which was then used to assess expression levels between the sample cohorts. The choice of control gene was important, as it was desirable that its expression

levels were constant irrespective of disease and similar to those of CD32, and that an RT-PCR product of similar size to that of CD32 could be designed.

Housekeeping genes are often used as control genes. They code for proteins involved in the normal maintenance of the cell and their expression level is expected to be consistent, for a given cell type, across all samples tested, irrespective of disease. It is desirable that the expression level of the housekeeping gene is similar to the expression level of the gene of interest. This allows the exposure of both bands on autoradiography film to be in the linear phase, preventing over-estimation of the less intense band due to saturation of the film by the more intense band.

Pseudogenes of the control gene may be present in the genome and these must not be amplified. Pseudogenes are non-allelic genomic copies of normal genes which are mutated, preventing functional activity. They often lack intron sequences, and may have arisen from viral integration of reverse transcribed mRNA into the host genome, as they often have short poly(A) tails (Weaver & Hedrick, 1992). Therefore, if pseudogenes, of either test or control gene, are present the PCR primers may amplify the pseudogene from contaminating DNA, and the intensity of the band produced may therefore be more a reflection of the amount of DNA contamination in the sample rather than the expression level of the gene of interest. All primer sequences used in the work presented here were designed so that the PCR product spanned at least one exon-intron boundary to prevent amplification of contaminating DNA in RNA preparations.

Finally, the PCR product size should be similar between test and control genes. Smaller products are preferentially amplified in a PCR reaction and this effect is relative to the size difference (Gale *et al*, 1996), therefore, in a multiplex reaction it is important for the size difference between the two PCR products to be the minimum needed for adequate separation of the two bands.

The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as the control gene for these studies as its expression level was similar to those of CD32, and while there is a GAPDH pseudogene, the primers have been designed from a region of GAPDH which is non-homologous to its pseudogene (Lehmann *et al*, 2002). These primers produce a product of 471bp which enabled primers to be designed for CD32 to produce a product of 360bp which crossed exon/intron boundaries.

Reverse transcription and PCR reactions were carried out from platelet RNA samples as described in chapter 2 (2.2.6 and 7). PCR reactions were carried out using

GAPDH primers and CD32 primers (Table 7.4), at an annealing temperature of 62°C. Ten pmol of one primer from each primer pair was added to an end-labelling reaction as described in chapter 2 (2.2.8). Twenty two cycles of PCR were carried out and samples were loaded onto 6% denaturing polyacrylamide gels (32 x 40 x 0.4cm) as described in chapter 2 (2.2.5) and electrophoresed at 1500V for 4 hours. Gels were exposed to autoradiography film prior to densitometry (GS300 Scanning Densitometer, Hoefer Scientific Instruments, San Francisco, CA). The densitometry results for CD32 were divided by the result from GAPDH to obtain a ratio. Statistical analysis was performed using the non-parametric Wilcoxon signed ranks test with SPSS for windows version 11.5.0.

7.4.3 Results

Platelet RNA from 52 ET patients, 8 patients with a reactive thrombocytosis, 11 PV patients and 10 normal controls was studied by multiplex SQ-RT-PCR. Two bands were produced after visualisation on autoradiography film. The GAPDH PCR product was 471bp and the CD32 PCR product 360bp. The intensity of the GAPDH PCR product was consistent across most samples tested, suggesting that GAPDH expression was similar for each sample. The intensity of the CD32 PCR product varied between samples indicating that CD32 was differentially expressed in different samples (Figure 7.9). The intensity of each band was quantified by densitometry and for each sample a ratio of CD32 to GAPDH expression was obtained by dividing results from the CD32 band by the results from the GAPDH band and multiplying by 100. Samples were analysed in duplicate and the average of the two results used for comparison.

The median CD32:GAPDH ratio from the 10 normal control samples was 1 (range <1-3) (Figure 7.10, Table 7.5). The expression of CD32 was often very low in normal control samples compared to GAPDH, therefore where the expression of CD32 was too low to accurately measure without saturation of the GAPDH band, the expression ratio was designated <1. The median CD32:GAPDH ratio for the 52 ET patients was 21 (range 2-73), and this was significantly higher than results obtained from haematologically normal controls ($p=0.005$). The CD32:GAPDH ratios obtained from the 2 samples used to generate the RDA products were consistent with these results. The normal control used in the RDA reaction had a CD32:GAPDH ratio of 1 compared to 12 for the ET patient with monoclonal myelopoiesis. Results from the other patient groups studied also showed a statistically

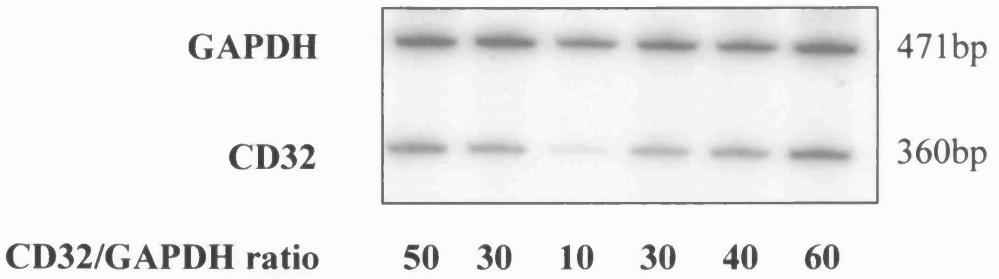

Figure 7.9
Multiplex semi-quantitative, radiolabelled RT-PCR for analysis of differential gene expression.
Results from six ET patient samples are shown with the corresponding CD32:GAPDH ratio.

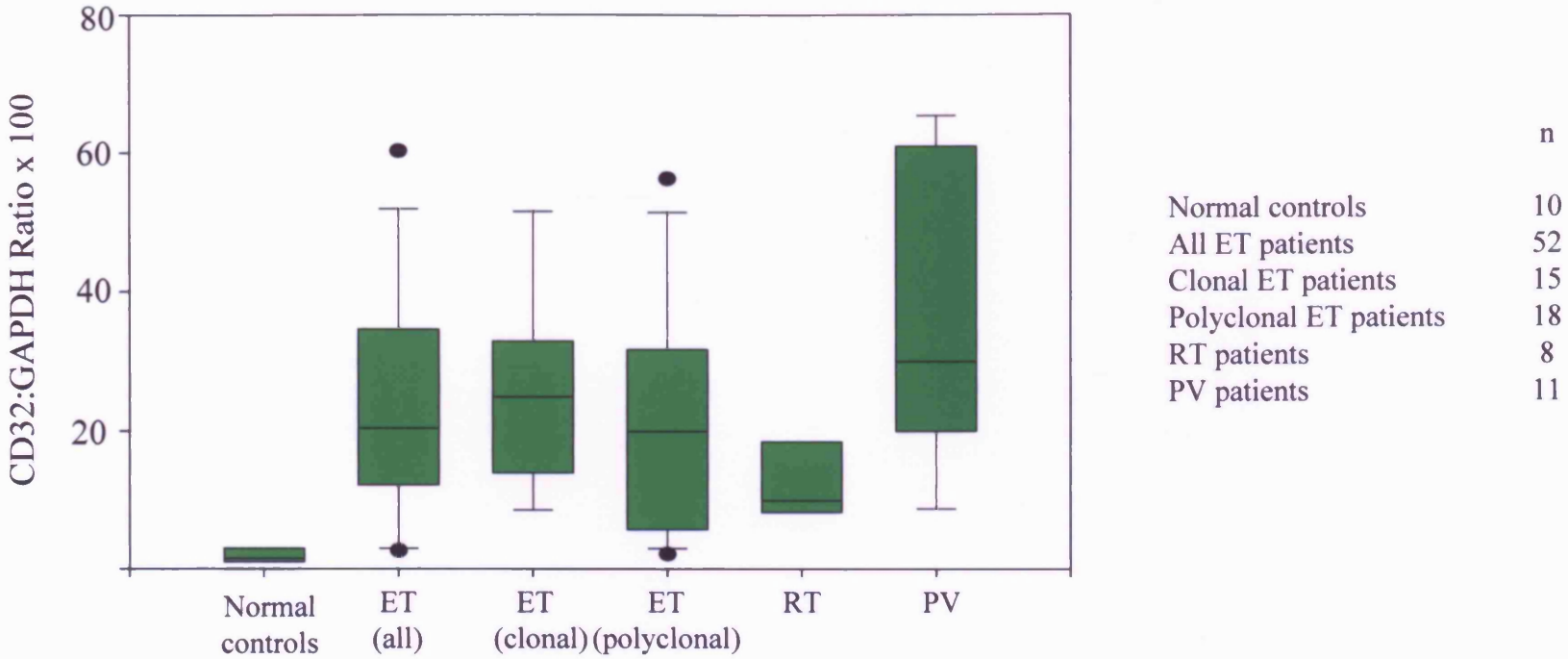

Figure 7.10
CD32:GAPDH ratios of each sample group.
The boxes represent interquartile range, bars represent the 10th and 90th percentiles and dots represent outlying results. The median of the results is represented by the horizontal bar within each box. RT = patients with a reactive thrombocytosis.

	n	Median CD32/GAPDH ratio x100	Range	p value
ET	52	21	2-73	0.005
(Clonal ET	15	25	8-60	0.005)
(Polyclonal ET	18	23	2-73	0.007)
RT	8	10	2-25	0.012
PV	11	30	7-63	0.008
Normal controls	10	1	<1-3	

Table 7.5
The CD32:GAPDH ratios for each of the patient groups studied.
p values are for comparisons between the normal control group and each patient group.

significant difference in CD32:GAPDH ratios compared to that obtained from the normal control group. PV patients had a median CD32:GAPDH ratio of 30 (range 7-63), and patients with a reactive thrombocytosis had a median ratio of 10 (range 2-25). The p values for comparison to the normal control group were 0.008 and 0.012 respectively. Therefore, the expression of CD32 was significantly increased in platelet mRNA obtained from 52 ET patients, 11 PV patients and 8 patients with a reactive thrombocytosis compared to haematologically normal controls.

Clonality data was available on 33 of the 52 ET patients, the remaining 19 were uninterpretable by XCIP analysis due either to a constitutively imbalanced pattern or age-related skewing. Of the 33 interpretable patients, 15 had clonal myelopoiesis and 18 had polyclonal myelopoiesis. The median CD32:GAPDH ratio for the ET patients with clonal myelopoiesis was 25 (range 8-60) and with polyclonal myelopoiesis was 23 (range 2-73). There was no significant difference between the clonal and polyclonal groups (p=0.281). Neither was there a significant difference when each group was compared to PV patients (clonal ET patients compared to PV patients p=0.594, polyclonal ET patients compared to PV patients p=0.173). However, the difference between CD32:GAPDH ratio results in clonal ET patients and patients with a reactive thrombocytosis did reach statistical significance (p=0.025), as did the difference between the CD32:GAPDH ratio with PV patients and patients with a reactive thrombocytosis (p=0.036).

7.5 CD32 polymorphism analysis

A G to A nucleotide polymorphism at position 507 of the CD32 gene has been shown to correlate with the incidence of thrombotic complications in heparin-induced thrombocytopenia (HIT). A G at position 507 codes for an arginine (R) at codon 131 of the CD32 protein, an A at position 507 leads to a histidine (H) at codon 131 (Clark *et al*, 1989; Warmerdam *et al*, 1990). A low affinity Fcγ receptor is produced when R is present at codon 131 and a high affinity Fcγ receptor when H is present, as demonstrated by binding studies in leucocytes (Warmerdam *et al*, 1991). The presence of the low affinity receptor (R131) in HIT led to inefficient removal of heparin/PF4/immuno-complexes, which in turn

led to prolonged activation of platelets and endothelial cells and caused an increase in thrombotic complications (Carlsson *et al*, 1998).

The RDA studies had identified an increase in CD32 mRNA expression in ET patients compared to normal controls, suggesting that CD32 is important in ET pathogenesis, and it was therefore of interest to screen ET patients for the G507A polymorphism. Neutrophil DNA obtained from 51 ET patients was analysed by PCR with a mismatch primer which created a *Bst*UI cutting site in products from G alleles to enable restriction fragment length polymorphism analysis. PCR was carried out using 35 amplification cycles and R131H primers (Table 7.3) at an annealing temperature of 66°C using Bioline reagents, as described in chapter 2 (2.2.7). Enzyme digestion reactions contained 10µl PCR product and 5U *Bst*UI in buffer 2 (NEB) as described in chapter 2 (2.2.11). The enzyme digestion reactions were incubated at 60°C for 17 hours before being electrophoresed through 3% agarose gels and visualised by ethidium bromide staining. Uncut PCR product was 231bp and the presence of G alleles at position 507 of the CD32 gene led to digestion with B*st*UI generating bands of 202bp and 29bp. A alleles remained uncut at 231bp.

Data on a cohort of 283 normal control samples of UK origin had been reported for the G507A polymorphism and the percentage of each genotype was shown to be 32% homozygous GG (90/283), 46% heterozygous GA (131/283) and 22% homozygous AA (62/283) (Karassa *et al*, 2003). The percentage of each genotype in the cohort of 51 ET patients was 41% homozygous GG (21/51), 41% heterozygous GA (21/51) and 18% homozygous AA (9/51). There was therefore, no significant difference between ET patients and the published cohort of normal control samples ($p > 0.1$ by χ^2 analysis).

Of the 51 ET patients, 15 had clonal myelopoiesis and 18 had polyclonal myelopoiesis by XCIP analysis, the remaining 18 patients were uninterpretable by XCIP analysis due to either a constitutively imbalanced XCIP or due to age-related skewing. The percentage of each genotype for the 15 patients with clonal myelopoiesis was 47% homozygous GG (7/15), 53% heterozygous GA (8/15) and 0% were homozygous AA. Of the 18 patients with polyclonal myelopoiesis 41% were homozygous GG (9/18), 36% were heterozygous GA (8/18) and 23% were homozygous AA (5/18) (Figure 7.11). Allele frequencies for the G allele in normal controls, all ET patients, clonal ET patients and polyclonal ET patients were 0.55, 0.62, 0.73 and 0.60 respectively (Figure 7.11).

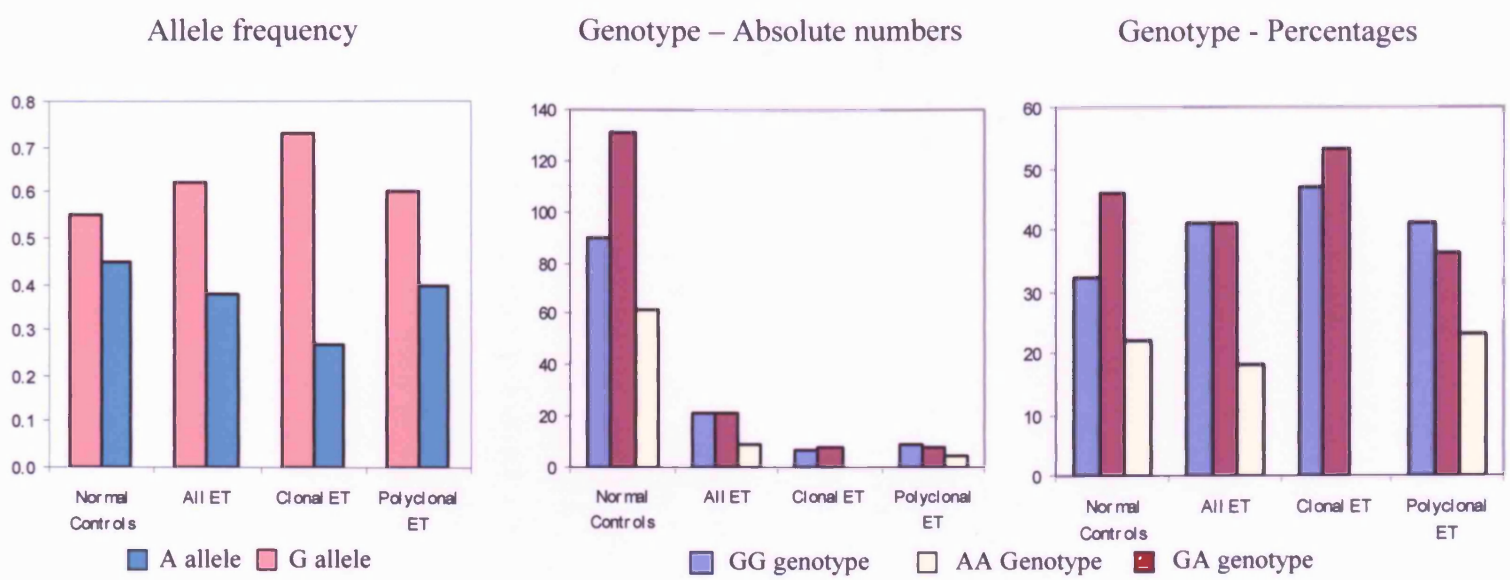

Figure 7.11

Genotype and allele frequencies of the G507A polymorphism in the CD32 gene in ET patients and normal controls.

Results for the 283 normal controls used were taken from Karassa *et al* (2003). 51 ET patients were screened, and of these 15 had clonal myelopoiesis and 18 had polyclonal myelopoiesis by XCIP.

No difference in genotype or allele frequency could therefore be demonstrated between normal controls and ET patients with polyclonal myelopoiesis. However, in ET patients with clonal myelopoiesis the A allele appears to be under-represented as none of this group were homozygous for the A allele. Also, the percentage of GG homozygotes was increased in the clonal group compared to normal controls (47% and 32% respectively). However, this difference did not reach significance by χ^2 analysis ($p>0.05$).

7.6 Discussion

RDA allows a global approach to be adopted to investigate differences in gene expression. It can identify both rare and novel genes which are differentially expressed between two groups, unlike other strategies which may only identify expression differences in known genes or gross changes in expression levels. In terms of platelet count, ET is defined by counts in excess of $600x10^6$/ml, which represents just one doubling of normal platelet levels, therefore subtle differences in expression levels of key genes may be important in its pathogenesis. RDA is highly sensitive and can therefore potentially identify subtle changes in gene expression levels, making it appropriate for investigating ET.

Platelet mRNA from an ET patient with monoclonal myelopoiesis and a haematologically normal control was used to create tester and driver for the RDA procedure. Driver and tester were hybridized using an excess of driver and amplified by selective PCR to produce selected difference products. These difference products were then hybridised with a greater excess driver to produce DP2 products. The RDA technique can produce false positive clones, and a number of screening procedures are required prior to establishing differential expression between patient groups. The RDA procedure and cloning of DP2 products was carried out by Dr. R. E. Gale and therefore only the screening of these clones is discussed within this section.

The 48 clones from the forward nM2 and reverse mN2 reactions were hybridized to nM2, mN2, M_{rep} and N_{rep} to establish the likelihood that each clone was differentially expressed. This was necessary because false positive clones could be generated because genes which are expressed at a very high level in both tester and driver may not be adequately removed by dilution between each round of differential amplification. From the

dot blot analysis, 23 clones were selected that appeared likely to be from genes differentially expressed between the monoclonal ET patient and the normal control sample.

Virtual northern blot analysis was used to screen the clones selected by dot blot by hybridization of PCR products from candidate clones to M_{rep} and N_{rep}. A problem with the dot blot technique is the difficulty of accurately scoring each clone, as non-specific binding of probes to dots can lead to all dots having a background level of positivity. Figure 7.6 demonstrates this problem by highlighting a positive and negative result in a probed blot with high background levels. Virtual northern analysis identified 13 of the 23 candidate clones as being differentially expressed between M_{rep} and N_{rep}. Sequencing was then carried out on these 13 clones to identify which genes they were fragments from.

Nine of the 13 clones were fragments from 3 mitochondrial genes (12S rRNA, Cytochrome C oxidase subunit II and Cytochrome b) and 4 clones were fragments of 4 genomic genes (RANTES, CD32, FLP and NRD1). The high number of mitochondrial RNA (mtRNA) transcripts identified in DP2 probably represent false positives due to high levels of mtRNA in circulating platelets. In a gene profile analysis of normal platelet, using serial analysis of gene expression (SAGE) tags to identify expressed sequences, 89% of SAGE tags represented mtRNA transcripts, and these were enriched for 16S and 12S rRNA (Gnatenko *et al*, 2003). Six of the 9 clones identified as fragments from mtRNAs were 12S rRNA. The high level of mitochondrial transcripts in platelets probably reflects persistent transcription from the mitochondrial genome in the absence of nuclear-derived transcripts.

As the starting material for RDA was platelet mRNA from one clonal ET patient and one normal control, it was likely that some genes would be expressed at different levels due to biological variation rather than disease specificity. This problem may be reduced by using pooled RNA from patients and controls, but it is unlikely to be eradicated completely. To screen for inter-individual differences in gene expression, platelet RNA from 3 ET patients with clonal myelopoiesis and 3 normal control samples was used for variable cycle number RT-PCR. More PCR product was obtained for RANTES, CD32 and FLP from ET patients than normal controls by visual examination of bands on agarose gels. However, no difference in the intensity of the bands was observed for the mitochondrial genes, further suggesting that they were false positives due to the high levels of mitochondrial transcripts in platelets.

Of the three genes shown to be overexpressed in ET patients by variable cycle number RT-PCR, CD32 appeared to be the most likely to be involved in ET pathogenesis.

CD32 is the only Fcγ receptor present on platelets (Ravetch & Kinet, 1991) and is involved in signal transduction via its immune receptor tyrosine activation motif (ITAM). It has been implicated in platelet activation via a number of pathways. The most well characterised of these is the association between CD32 and the vWF/GpIb-IX-V activation pathway. CD32 has been shown to physically associate with the vWF/GpIb-IX-V complex using co-immunoprecipitation in yeast two-hybrid experiments (Sullam *et al*, 1998), and anti-GpIb antibodies could completely inhibit platelet aggregation in viridans group streptococci known to induce aggregation via CD32 (Sullam *et al*, 1998; Sun *et al*, 1999). Also, platelets stimulated with vWF showed tyrosine phosphorylation of CD32 whereas unstimulated platelets did not (Canobbio *et al*, 2001). These data suggest that vWF/GpIb-IX-V dependent platelet activation involves CD32 mediation. Using flow cytometric analysis, increased platelet activation has been described in all MPDs, with a significant increase of surface CD62p (p-selectin, which migrates from the α-granules to the platelet surface during activation) and platelet-leucocyte conjugations in comparison to haematologically normal controls, suggesting that activation may be important for disease pathology, although this may be a secondary event leading to an increase in complications (Villmow *et al*, 2002).

CD32 was shown to have an approximately 20 fold increase in transcript level in platelets from ET patients compared to platelets from normal controls. Transcript levels in clonal ET patients were also significantly higher than reactive thrombocytosis patients. However, transcript levels were not significantly different between ET and PV patients, or between the whole ET cohort and patients with a reactive thrombocytosis. The range of results for each patient group was relatively wide (the widest range was 2-73 in ET patients), resulting in a large overlap of results between groups. This makes drawing conclusions regarding the significance of differences between groups difficult, as the number of patients used in this study was relatively small. However, the range of results was narrow for the normal control group (<1-3), suggesting that CD32 expression is under tight regulation in the normal situation. In ET and PV patients, a disease related variable increase in platelet activation may account for the wide range of results observed. Whilst the differences between some patient groups were statistically significant, this may be a reflection of the low number of patients in each group. This is illustrated by the finding that whilst no difference could be demonstrated between results from polyclonal patients compared to either clonal patients or those with a reactive thrombocytosis, clonal patients

were significantly different from those with a reactive thrombocytosis. Therefore, whilst it is evident that ET patients have raised CD32 transcript levels compared to normal individuals, it is unlikely that a raised CD32 level could be used to distinguish ET patients from other cMPDs or patients with a reactive thrombocytosis. As no significant difference in CD32 levels could be demonstrated between clonal ET patients at higher risk of thrombotic complications, and polyclonal patients at lower risk of thrombosis, this data alone does not support the hypothesis that thrombotic events in ET may be due to a general increase in platelet activation caused by an increase in CD32 expression.

CD32 has been implicated in increased thrombotic risk in relation to a polymorphism in the coding region of the gene. A G509A polymorphism, which leads to an arginine to histidine amino acid change at codon 131, has been correlated to thrombotic complications in HIT. G alleles lead to the build-up of immune complexes in the circulation and ultimately thrombotic events. No significant change in genotype or allele frequency could be demonstrated between 51 ET patients and the reported levels of 283 normal controls from a UK population (Karassa *et al*, 2003). However, clonal patients appeared to have an increase prevalence of G alleles with an allele frequency of 0.73 compared to 0.55 for controls. At the genotype level, no AA homozygotes were present in the clonal cohort. Although this could indicate that at least some of the increase in thrombosis seen in patients with clonal myelopoiesis may be partly due to the R131H polymorphism in CD32 and platelets from these patients may be primed for activation, the clonal cohort is small, containing only 15 patients, and the altered genotype compared to normal controls did not reach statistical significance. Further studies with a larger cohort of clonal and polyclonal patients is necessary to establish the significance of the R131H polymorphism in thrombotic complications associated with ET.

In conclusion, RDA has successfully identified 3 genes which appear to be differentially expressed between platelet mRNA obtained from ET patients with clonal XCIP and haematologically normal controls, RANTES, FLP, and CD32. After further investigation, the CD32 gene was shown to be expressed at a significantly higher level in ET patients, where a median increase in expression levels of approximately 20 fold was observed. However, platelet mRNA CD32 expression levels were not significantly different between ET, PV and patients with a reactive thrombocytosis, nor between ET patients with clonal or polyclonal myelopoiesis, suggesting that CD32 levels are not useful as a diagnostic marker. Further investigation of the other genes which were shown to be

differentially expressed between platelets from ET patients with clonal myelopoiesis and normal controls (RANTES and FLP) in larger patient groups is required as they may be important in the pathogenesis of ET.

Chapter 8

Conclusions

The chronic myeloproliferative disorder ET is a heterogeneous disorder with unknown aetiology. It is characterized by sustained thrombocytosis and MK hyperplasia, and diagnosis requires exclusion of other cMPDs and reactive thrombocytosis. While the clinical course of ET is benign and often includes long asymptomatic periods, these periods can be punctuated with life-threatening haemorrhagic or thrombotic complications. Risk factors for these complications are poorly understood and only old age (>65 years), excessive platelet counts (>1500x10^6/ml) and a previous history of thrombotic or haemorrhagic complications are well defined risk factors (Cortelazzo *et al*, 1990; van Genderen *et al*, 1994). As some platelet lowering agents such as hydroxyurea and busulphan may be leukaemogenic, and others such as anagrelide may not influence long-term thrombotic rates and induce transformation to cIMF (Sterkers *et al*, 1998; Green *et al*, 2004; Randi *et al*, 1999), it is important to establish risk for these complications to enable better assessment of the need for platelet lowering treatment. In this thesis the pathophysiology of ET has been investigated by longitudinal assessment of clonality in female ET patients by XCIP, the examination of specific candidate genes for mutations or polymorphisms which may be used as molecular markers, and an evaluation of global gene expression in platelet mRNA to try to identify genes which may have a role in the disease.

As no disease-specific markers have been conclusively identified for ET, clonality cannot be measured directly. However, an indirect PCR-based method using X-chromosome inactivation patterns has allowed clonality to be assessed in a number of female ET patients. It has been shown by several groups that ET patients can have either clonal or polyclonal myelopoiesis, and that ET patients with clonal myelopoiesis are at greater risk of thrombosis than those with polyclonal myelopoiesis (Harrison *et al*, 1999a; Chiusolo *et al*, 2001; Shih *et al*, 2002; Vannucchi *et al*, 2004). However, it is not known whether clonality can change during the course of the disease, and the question remains whether patients with polyclonal myelopoiesis progress to a clonal disorder with an associated increase in thrombotic risk. To address this question, sequential samples were collected from ET patients to investigate changes to clonality status over time.

Some of the initial patient samples had been collected and assayed for clonality status previously in the laboratory, using radiolabelled XCIP assays. It was desirable to avoid the use of radioactivity in the laboratory as the ^{32}P radioisotope used in this assay is a mutagen requiring specialist handling techniques and highly regulated disposal and also requires the use of a Geiger counter for detection. It has a short shelf life due to its 14.5 day

half-life and the visualization and densitometry required for quantitative analysis are time consuming and labour intensive. A fluorescent primer label was therefore investigated for reproducibility and comparison to previous clonality results, as fluorescent labels overcome most of the problems associated with radioactive isotopes.

Basic conditions for the DNA-based HUMARA XCIP assay were established and results from 25 DNA samples, with previous radioactive data which covered the whole range of possible XCIP results, were compared with fluorescent data. The median difference between radioactive and fluorescent results was 1% (range 0-4%). Having established reproducibility and reliability for the fluorescent HUMARA, XCIP results from purified neutrophils and T-cells obtained from 18 ET samples were compared with previous radioactive data and the median difference was 3.1% (range 0-22%) with correlation coefficients $r=0.980$ and $r^2=0.960$. The IDS RNA XCIP assay was also investigated and basic conditions established. As the IDS assay does not use methylation sensitive restriction enzymes the digest is carried out after the PCR amplification and it was found that the salt concentration of the digest reaction inhibited loading of DNA onto the capillary of the genetic analyser used to visualize and quantify the results. Therefore, the excess salt was remove from the reaction using a DNA clean-up kit. Results from RNA obtained from neutrophils, T-cells and platelets from 10 ET patients were compared with previous radioactive data and the median difference between results was 2% (range 0-10%). Results obtained using the fluorescent DNA HUMARA and RNA IDS assays were also compared. The median difference between results was 6% (range 0-22%) and correlation coefficients were $r=0.95$ and $r^2=0.90$. This demonstrated that the fluorescent primer label gave equivalent results to the radioactive primer label for both DNA and RNA assays, and that clonality results were similar irrespective of whether DNA or RNA was used for the assay.

The fluorescent XCIP assays were used to study clonality status during the course of disease in 22 ET patients. Of these patients, 8 had clonal myelopoiesis at the time of first test, had a median age of 55 years, and up to 4 samples were collected from each patient over a median of 52 months (range 5-97 months). Neutrophil results changed by a median of 1.5% (range 0-16%) and all patients remained clonal by HUMARA at the end of the follow-up period. The 14 polyclonal patients had a median age of 49 years, and up to 5 samples were collected from each patient over a median of 54 months (range 10-102 months). Neutrophil results varied by a median of 6% (range 0-25%) from first test results and all polyclonal patients remained polyclonal by HUMARA after a median 54 months

(range 10-102 months). The first test samples were not necessarily at diagnosis in these patients and the median time since diagnosis for the polyclonal group was 78 months (range 45-188 months). This data shows that in this cohort of ET patients with polyclonal myelopoiesis, clonality is stable and can remain unchanged for up to at least 188 months.

Polyclonal ET has been confirmed in this study and also by a number of other groups (Chiusolo *et al*, 2001; Harrison *et al*, 1999a; Shih *et al*, 2002; Vannucchi *et al*, 2004), but it is possible that ET patients may have polyclonal myelopoiesis, but clonal thrombopoiesis with the clonal population therefore being restricted to the platelet lineage. EL Kasser *et al* (1997) reported that XCIPs from 3 out of 31 ET patients showed monoclonal pattern of expression by RNA analysis of platelet samples, but polyclonal pattern with neutrophils. This suggested that the clonal population derived from an altered stem cell can be restricted to the MK lineage. As platelets are anucleate RNA assays are required for studying their clonality.

In this study, 9 patients were investigated for XCIPs of their T-cells, neutrophils and platelets by RNA assay and T-cells and neutrophils by DNA HUMARA. The mean difference between HUMARA and IDS assay results from T-cells and neutrophils of this initial test cohort was 7.2%, and the median difference was 6% (range 0-22%). While the difference between platelet RNA duplicates was greater than 10% in 3 cases all patients investigate had either balanced XCIPs in neutrophils and platelets or imbalanced XCIPs in neutrophils and platelets. The only difference between RNA and DNA results sufficient to affect the clonality result was seen with T-cells from patients in the follow-up study. Three out of 4 patients with a balanced T-cell XCIP by DNA HUMARA had an imbalanced XCIP using RNA assays. This was probably due to contamination of the purified T-cell population by clonal platelets. However, all patients polyclonal by DNA or RNA neutrophil assay were also polyclonal by platelet RNA assay. Therefore, it is unlikely that lineage restriction affects a significant proportion of clonality results for ET patients, and while RNA assays may provide more information about clonality status, particularly where patients are uninformative for DNA assays, if an imbalanced XCIP is evident in purified T-cell samples, contamination from platelets should be ruled out before excluding the sample due to a constitutively imbalanced XCIP.

This longitudinal study of clonality shows that ET with polyclonal myelopoiesis is a stable subgroup of ET patients using XCIP analysis with either RNA or DNA. It may therefore, be useful to routinely assess clonality status in ET patients as polyclonal patients

have a lower risk of thrombotic events. It may be possible to restrict the amount of platelet reducing drugs received by polyclonal patients on this basis, and a retrospective analysis of patients from one centre has shown that their polyclonal patients did receive less cytoreductive therapy (Briere *et al*, 1999). However, this data can never be used in isolation, and all factors must be taken into account before such a decision is taken.

Polyclonal ET is likely to be due to mutations that lead to an excessive external drive to megakaryocytopoiesis. Mutations or polymorphisms in cytokines or growth factors that regulate thrombopoiesis may be important for disease pathogenesis, therefore the cytokines TPO, TGFβ1 and IL-6 were investigated for previously described or novel mutations/polymorphisms.

The possible role of TPO in the pathogenesis of ET was investigated by mutation analysis of its 5'UTR. TPO is the prime regulator of thrombopoiesis and interacts specifically with its receptor Mpl on the surface of all cells of the MK lineage to promote proliferation, differentiation and, ultimately, platelet production. TPO production is thought to be constitutive and circulating levels in normal individuals controlled by TPO binding to Mpl and internalization in MK and platelets. Therefore, circulating TPO levels are regulated by, and inversely proportional to, platelet mass (Kuter & Rosenberg, 1995). However, TPO levels are normal or elevated in ET, suggesting dysregulation of either TPO production or TPO removal. The 5'UTR of the TPO gene contains 8 AUG transcription initiation sites, the first 7 of these cause suppression of transcription initiation from the physiological start site, AUG 8, by production of peptides from short ORFs. The most significant initiation site is AUG 7 as the ORF from this site extends beyond the physiological start site, blocking access to AUG 8. Four mutations have been described in HT which reduce this transcriptional suppression leading to an increase in TPO expression and subsequent thrombocytosis, all four mutations affect suppression from AUG 7 (Ghilardi & Skoda, 1999; Jorgensen *et al*, 1998; Kondo *et al*, 1998; Wiestner *et al*, 1998; Ghilardi *et al*, 1999). As the mutation described by Weistner *et al* (1998) had previously been investigated in ET patients, the remaining 3 mutations were investigated by RFLP analysis of neutrophil DNA from 50 ET patients. None of the mutations described in HT were present in the TPO 5'UTR of ET patients. To further investigate the 5'UTR, SSCP was carried out to screen for previously undescribed mutations. While an alteration was identified, 10bp downstream from the 5' end of exon 3 and 65 bp upstream of AUG 7, it was considered to be a common polymorphism as it did not appear to alter splicing of the

5'UTR, did not introduce a start site or stop site and was present in normal controls at a similar gene frequency and with similar genotype percentages.

This data does not rule out a role for TPO in the pathogenesis of ET. Only the 5'UTR of the TPO gene was investigated for mutations. Other mutations may be present, either in the TPO gene promoter sequences which may lead to an increase in gene expression, or in the coding region of the TPO gene which may increase the half-life of the mRNA transcript or the protein. Both these instances would be expected to lead to an increase in the circulating levels of TPO and may be worth further investigation. Also, mutations have been described in c-Mpl which may affect circulating TPO levels by reducing the binding efficiency of Mpl to TPO, thereby reducing the removal of TPO from the circulation (Moliterno *et al*, 2004; Ding *et al*, 2004). However, no c-mpl mutations have yet been shown to be present in ET patients (Kiladjian *et al*, 1997; Taksin *et al*, 1999; Randi *et al*, 2004). These studies were small and only included a total of 20 ET patients, therefore investigations in a larger cohort may be worthwhile.

TGFβ1 is a negative regulator of thrombopoiesis, but its circulating levels are elevated in ET (Harrison *et al*, 1999b; Kuroda *et al*, 2004; Lev *et al*, 2002). Four polymorphisms in the TGFβ1 gene, which affect its level of expression have been described and shown to be risk factors in disease and for severity of disease (Grainger *et al*, 1999; Yamada *et al*, 1999). Two of these mutations are in the gene promoter and 2 are in the coding region which encodes the signal peptide and are likely to effect protein trafficking to the cell surface. A shift in the gene frequency or genotype of the polymorphisms in ET patients could account for the reported increase in TGFβ1 levels. Seventy five patients were investigated for the four TGFβ1 polymorphisms by RFLP analysis. However, no significant difference could be shown between patients and 100 normal control subjects in either genotypes or gene frequencies.

Undescribed polymorphisms or mutations may be present in the TGFβ1 gene coding or non-coding regions which may affect its release into the circulation. Therefore, a mutation screen of the entire promoter and coding regions of the TGFβ1 gene may be of value. However, as the major store of TGFβ1 is the α-granules of MK and platelets, it may simply be that the increased MK and platelet mass in ET patients accounts for the increase in serum/plasma TGFβ1.

ET patients may be resistant to the inhibitory effects of TGFβ1. A 10 fold higher platelet lysate concentration was required to inhibit CFU-MK derived from ET BM CD34+ cells than that needed for CFU-MK derived from normal BM CD34+ cells (Zauli *et al*, 1993). Reversal of all inhibition occurred by addition of anti-TGFβ1 antibodies, suggesting that TGFβ1 was the main factor responsible for the inhibition. This data was confirmed by a second group, which linked insensitivity to TGFβ1 to a decrease in the expression of the downstream effector molecule Smad4 (Kuroda *et al*, 2004). It would be of value to investigate Smad4 in some detail, by a full investigation of expression levels at both the mRNA and protein levels in BM MK or platelets, and by mutation analysis of both the promoter and coding gene sequences in a large cohort of ET patients and controls.

IL-6 is able to stimulate the production of platelets and can augment their maturation and proliferation in combination with other factors, and IL-6 levels have been shown to be dysregulated in ET by a number of groups (Alexandrakis *et al*, 2003; Uppenkamp *et al*, 1998; Harrison *et al*, 1999c). A polymorphism is present in the promoter region of the IL-6 gene, a G to C at nucleotide -174, and presence of the G allele (the higher producer allele) has been shown to be a risk factor for GVHD after BM transplant and other disorders associated with high levels of IL-6 (Cavet *et al*, 2001; Foster *et al*, 2000; Fishman *et al*, 1998). If G alleles were more prevalent in ET patients, this would suggest that IL-6 may be involved in augmenting the platelet product in these patients. However, using RFLP analysis to investigate the genotype and allele frequency for the IL-6 polymorphism in 75 patients and 100 normal controls, no significant difference could be demonstrated between the two cohorts, therefore this polymorphism is unlikely to be significant in the pathogenesis of ET. Other alterations to the promoter or coding regions of IL-6 may be important in ET and therefore it might be useful to screen the entire gene for novel mutations or polymorphisms.

Unfortunately, this study has been unable to demonstrate a link between ET pathogenesis and mutations or polymorphisms in either TPO, TGFβ1 or IL-6. These cytokines may still have a role in ET and their further investigation could help to understand the biology of ET.

As the pathogenesis of ET may be extremely complex an attempt was made to identify genes which were differentially expressed between ET patients and normal

controls. RDA was used because it can identify gross and subtle changes in mRNA levels without any sequence information, therefore both known and undescribed genes can be identified. The procedure was carried out on platelet mRNA from an ET patient with monoclonal myelopoiesis and a haematologically normal control sample.

Representations were prepared from cDNA from each sample by *Dpn*II digestion of the double stranded cDNA followed by PCR amplification. The representations were then used either as driver or tester in the hybridization which was carried out in an excess of driver. Two difference products (DP1) were generated by selective PCR: the forward reaction nM1 (N_{rep} driver, M_{rep} tester – enriched for sequences only, or more highly expressed, in the monoclonal ET patient sample) and the reverse reaction mN1 (M_{rep} driver, N_{rep} tester – enriched for sequences only, or more highly expressed, in normal control sample). A second round of hybridization was carried out to further enrich for differentially expressed genes using DP1 products and a greater excess of driver. DP2 products were then cloned into the TOPO-2 vector (Invitrogen) and 48 clones isolated for both the forward (nM2) and reverse (mN2) RDA reaction. The RDA DP2 clones were provided by Dr R. E. Gale.

As the rate of false positives produced by RDA is high, the clones were screened by hybridization to both initial representations and both forward and reverse subtractions using dot blot analysis. Of the 96 clones screened, 23 appeared likely to be from differentially expressed genes. Each clone selected from the dot blots was further screened by hybridization to each representation using virtual northern analysis. Thirteen of the 23 clones were selected for sequencing as they appeared to be present in the representations at variable levels. These 13 clones were fragments from 4 genomic genes: RANTES, CD32, FLP, NRD1, and three mitochondrial genes: 12S rRNA, Cytochrome C Oxidase subunit II and cytochrome b. Using variable cycle number RT-PCR, RANTES, CD32 and FLP appeared differentially expressed between ET patients with clonal myelopoiesis and normal controls. The mitochondrial genes were shown to be false positives as they did not appear differentially expressed. NRD1 was not further investigated as a quantifiable PCR product could not be produced. CD32 (FcγIIRA) is the only FcγR molecule expressed by platelets. It has been shown to associate with the vWF/GpIb-IX-V complex and mediate platelet activation and thrombus formation (Sullam *et al*, 1998; Sun *et al*, 1999). It was, therefore further investigated because it was thought to be the gene identified by the RDA procedure

which was most likely to be involved in ET pathogenesis or complications associated with ET.

Platelet RNA from 52 ET patients, 8 patients with a reactive thrombocytosis, 11 PV patients and 10 normal controls was studied by multiplex SQ-RT-PCR and comparison of CD32 mRNA levels to those of the housekeeping gene GAPDH. All patient groups were shown to have significantly increased CD32 mRNA expression compared to normal controls ($p<0.012$). Of the patient groups studied, patients with a reactive thrombocytosis had the lowest CD32 mRNA expression levels (median CD32 to GAPDH expression ratio 10, range 2-25), although this was not significantly different from the entire ET patient cohort, which had a median CD32 to GAPDH expression ratio of 21 (range 2-73). No significant difference was demonstrable between ET patients with a clonal myelopoiesis (median CD32 to GAPDH expression ratio 25, range 8-60) or those with a polyclonal myelopoiesis (median CD32 to GAPDH expression ratio 23, range 2-73), $p=0.281$. CD32 mRNA levels were also high in PV patients, which had a median CD32 to GAPDH expression ratio 30, range 7-63. CD32 mRNA expression was, therefore able to differentiate ET patients from normal controls (normal control median CD32 to GAPDH expression ratio was 1, range <1-3), and this may be useful in a clinical setting, however, while patients with a reactive thrombocytosis had lower levels of CD32 platelet mRNA than ET patients, in this small cohort the difference was not significant. Assessing the platelet CD32 mRNA expression levels in a larger cohort of reactive patients and PV patients would be useful to test whether a significant difference compared to ET patients could be established.

The method used to quantify differential expression of platelet CD32 mRNA was a semi-quantitative method which compared mRNA expression levels of CD32 to those GAPDH in a multiplex PCR reaction. The multiplex PCR may not be equally efficient at amplifying mRNA from both genes. A quantitative assay, such as real-time RT-PCR would avoid the need to use a multiplex assay and may give a more accurate comparison, which in turn may lead to a more significant difference in expression, particularly between reactive patients and ET. The investigation of CD32 expression at the protein level is important, as an increase in mRNA transcripts may not lead to an increase at the protein level. This has been shown with the PRV-1 gene, where mRNA levels are raised in PV patients, but not cell surface protein levels (Klippel *et al*, 2002). This may be because the PRV-1 gene transcript is unstable (Jelinek *et al*, 2004). It would also be useful to compare clinical data

from patients with high CD32 expression with those who have low CD32 mRNA expression to try to identify a level of CD32 expression which may be clinically significant. This has not been carried out to date as the differences between groups were not significant using SQ-RT-PCR. A comparison of the levels of platelet activation markers such as p-selectin, with CD32 levels in this cohort would be of interest. This may establish whether CD32 is raised due to a general increase in the activation state of platelets from some ET patients and might be, together with other factors, contributing to an increased risk of thrombosis. CD32 expression may be dysregulated in ET patients. This may be due to an increase in the rate of platelet production from each MK in ET patients, which may in turn lead to abnormal expression of platelet proteins.

RDA has been used successfully to identify genes which appear to have a disease specific alteration in mRNA expression levels. CD32 expression has been shown to be raised in ET patients compared to normal controls, as have two other genes, RANTES and FLP. Further investigation of RANTES, FLP (and NRD1, which was selected from RDA screening, but could not be investigated by variable cycle number RT-PCR as a clean PCR product could not be obtained) is required to fully understand their role, if any, in ET pathogenesis.

A G507A SNP in the CD32 gene had previously been shown to be a risk factor for thrombosis in HIT (Clark *et al*, 1989; Warmerdam *et al*, 1990). This polymorphism was investigated in neutrophils from 51 ET patients and compared to published control levels (Karassa *et al*, 2003). Whilst no significant differences between ET patients and controls, nor ET patients with clonal or polyclonal myelopoiesis could be demonstrated, a trend was identified between the presence of the G allele in clonal patients compared to polyclonal patients. Also, no clonal patients were homozygous for the A allele compared to 18% of patients with polyclonal myelopoiesis and 22% of normal controls. This difference may become significant in a larger cohort and may contribute to the increased risk of thrombosis in clonal patients. The clonal ET cohort contained only 15 patients, and the polyclonal cohort 18 patients, therefore it may be of interest to compare the levels of CD32 expression with G507A genotype in a larger number of patients. Also, an investigation for mutations in the promoter and coding regions of CD32 may reveal more mutations and/or polymorphisms which may be important in either the pathogenesis of ET, or in predicting risk of thrombotic events.

A recent discovery has been made that may advance the understanding of the pathogenesis of ET and other cMPDs. An acquired G→T mutation at position 1848 in exon 12 of the Janus Kinase 2 (JAK2) gene has been described independently by 5 groups (Baxter *et al*, 2005; James *et al*, 2005; Kralovics *et al*, 2005; Levine *et al*, 2005; Zhao *et al*, 2005). JAK2 is a member of the janus kinase family of tyrosine kinases with a key role in signal transduction from multiple haemopoietic growth factor receptors including the EPO receptor and Mpl. The mutation causes phenylalanine to be substituted for valine at codon 617 of JAK2 (V617F). This codon lies in a JH2 pseudokinase domain which is thought to have auto-inhibitory action (Saharinen *et al*, 2000). The mutation therefore, is thought to produce a constitutive tyrosine phosphorylation activity. The mutation was present at high frequency in granulocytes from patients with PV; 434 patients were studied between the 5 groups and the mean percentage with the mutation was 85% (range 74-97%). In 92 patients with cIMF the median percentage of patients with the mutation was 46% (range 35-57%). It was also present in ET patients, but at a lower frequency, the mean occurrence in a total of 280 ET patients being 28% (range 22-57%). The mutation is thought to be acquired as it could not be found in T-cells and non-haemopoietic tissue. The percentage of patients with JAK2 mutations may be higher than these reports as most studies used sequencing to identify the mutation. However, Baxter *et al* (2005) found 6 out of 51 ET patients had the mutation using sequencing, but when they used a more sensitive allele-specific PCR method they found that 23 of the 45 patients negative by sequencing were positive by allele-specific PCR. This suggests that the mutation level in patients may be variable, and this may be significant for disease pathogenesis. Zhao *et al* (2005) have shown that the level of the mutation seen with genomic DNA was significantly lower than that observed for cDNA, suggesting that expression of the JAK2 mutant cDNA may be higher than the normal gene, therefore investigating JAK2 mutation from RNA may be preferable. This would also enable analysis of platelets to investigate the possibility that the mutation may cause differential production of mature end-stage cells in different lineages. It also raises the question as to whether the level of JAK2 mutation changes during the course of the disease, and this could be addressed by a longitudinal study of multiple samples taken at different time points throughout the disease. Comparison of the presence of the JAK2 mutation with other factors which may be involved in ET pathogenesis, such a clonality would also be of interest. As well as addressing these questions it would be useful to investigate the actions of JAK2 inhibitors on cultured primary cells in an attempt to identify

molecules which may suppress the activity of mutant JAK2 and assess the possibility of using these molecules as treatment for cMPD patients who possess the V617F mutation.

The work presented in this thesis has shown that ET with polyclonal myelopoiesis is a stable subgroup of ET, and that CD32 expression is higher in platelets from ET patients than normal control subjects. It has also shown that specific mutations and polymorphism in TPO, TGFβ1 and IL-6 genes are not related to ET. This work also leads the way to further studies particularly regarding investigations of the genes identified by RDA, and identification of differences at the molecular level between ET patients with a clonal or polyclonal myelopoiesis.

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PUBLICATIONS ARISING FROM THIS WORK

Allen AJR, Gale RE, Harrison CN, Machin SJ, Linch DC.
Lack of Pathogenic mutations in the 5'-untranslated region of the thrombopoietin gene in patients with non-familial essential thrombocythaemia.
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